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Validated HPLC Method for Simultaneous Determination of Aripiprazole and Co-Administered Clonazepam in Spiked Human Plasma

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Abstract: A sensitive and selective high performance liquid chromatographic method was developed and validated for the simultaneous quantification of Aripiprazole and co-administered Clonazepam in spiked human plasma. Aripiprazole, Clonazepam and the internal standard Citalopram were extracted from plasma and analyzed on a reversed-phase C_{18} column using a mobile phase consisting of acetonitrile and 0.05M potassium dihydrogen orthophosphate (pH= 3.6) in a ratio of (40: 60, v/v). The ultraviolet (UV) detection was at 220 nm. The method was linear over concentration range (20-1000 ng mL^{-1}) for both drugs. The method is very simple and allows obtaining a very good recovery of the analytes.

Keywords: Aripiprazole; Clonazepam; plasma; HPLC; co-administered.

1 Introduction

Aripiprazole (ARI) (Fig.1), chemically is 7-(4-[4-(2,3-dichlorophenyl)-1-piperazinyl]butoxy)-3,4-dihydro-2(1*H*) quinolinone, molecular formula: $C_{23}H_{27}Cl_2N_3O_2$ and molecular weight: 448.39 [1]. ARI has been approved by FDA at the end of 2002 [2]. It is considered to act as a partial dopamine D_2-receptor agonist, partial serotonin (5-HT_1A) receptor agonist and (5-HT_2A) receptor antagonist [3, 4]. It is used in the management of schizophrenia and is also under investigation for bipolar disorder [1].

Clonazepam (CLO) (Fig.2) is a benzo diazepine derivative, chemically it is 5-(o-chlorophenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one [1]. CLO is a highly potent anticonvulsant, amnestic and anxiolytic. It has shown itself to be useful as a short-term adjunct to SSRI treatments in clinical depression and obsessive compulsive disorder, and this combination is superior to SSRI treatments alone [5]. The literature survey revealed analytical methods for the estimation of ARI with other drugs or in presence of its main metabolite in biological fluids, including HPLC methods [6-11] and capillary electrophoresis [12].

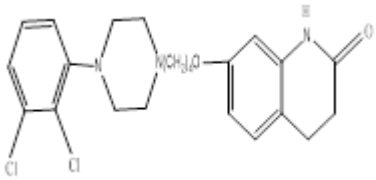

Fig. 1. Chemical structure of Aripiprazole

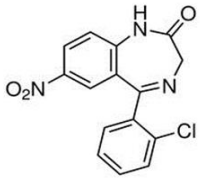

Fig. 2. Chemical structure of Clonazepam

2 Experimental

2.1 Apparatus

La chrom, Merck Hitachi series chromatographic system equipped with pump L-7110 and wavelength UV–VIS

detector L-7420 was used. Column X terra® RP-C_{18}column (150 mm×4.6 mm, 5 µm particle size i.d.) was from (waters corporation), Ultra-sound sonicator (Crest Ultrasonics, New York), pH meter equipped with combined glass electrode for pH adjustement (Jenway 3510,UK).

2.2 Reagents and materials

All chemicals used throughout the work were of analytical grade and solvents were of HPLC grade; acetonitrile, methanol and orthophosphoric acid (Riedel-dehaen, Sigma-Aldrich, Germany), potassium dihydrogen orthophosphate (ADWIC, Egypt).

2.3 Samples

Authentic samples: Aripiprazole (purity 99.87%) was kindly supplied by Bristol-Myers Squibb Company, Cairo, Egypt. Clonazepam (CLO) (purity 99.67%) was supplied by EIPICO (Egyptian International Pharmaceutical Industry Company), Egypt. The internal standard (I.S) Citalopram (purity 99.87%) was supplied by SEDICO Company, Egypt. The human plasma used was obtained from the holding company for biological products and vaccines (VACSERA, Egypt).

2.4 Standard solutions

Stock standard solutions (0.1 mg mL^{-1}) of ARI and CLO were prepared, daily in methanol. Working standard solutions of ARI and CLO (10.0 µg mL^{-1}) were prepared by suitable dilution of the stock solutions, respectively. Aliquots equivalent to (0.2, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 mL) were transferred from the ARI and CLO working solutions, separately and were diluted in 10-mL volumetric flasks with methanol to reach a concentration of (0.2-10.0 µg mL^{-1}). The internal standard solution was of concentration (0.1 mg mL^{-1}).

2.5 Sample preparation

0.7 mL of the frozen plasma was thawed at room temperature and was spiked with a fixed volume (100 µL) of the serially diluted solutions of ARI and CLO to obtain concentrations of (20-1000 ng mL^{-1}) and 100 µL of the I.S was added to give a concentration of (500 ng mL^{-1}).

2.6. Drug extraction

In this proposed method, several extracting solvents were tried; hexane, acetonitrile, methanol, perchloric acid, ethyl acetate and diethyl ether but the recoveries of the two drugs were 70-75%. A mixture of acetonitrile: methanol (ratio 1:1) was found to be optimal for precipitation of plasma proteins and for obtaining good recoveries of the drugs (recoveries were above 85%). Two mL of (acetonitrile: methanol, 1:1) was added to the spiked plasma then the mixture was vortexed for 2 min and centrifuged for 10 min at 4500 rpm. The upper layer was transferred to another clean tube and then filtered through a 0.45µm Millipore syringe filter. 10-µL of the supernatant was injected onto the liquid chromatographic system for analysis.

2.7 Chromatographic conditions

Using RP-C_{18} column(150 mm×4.6 mm, 5 µm particle size i.d.) and the mobile phase as a mixture of acetonitrile and 0.05M potassium dihydrogen orthophosphate (pH= 3.6 adjusted using orthophosphoric acid) in a ratio of (40: 60, v/v). The analysis was done under isocratic condition at a flow rate 1 mL min^{-1} and at room temperature using UV detector at 220 nm.

2.8 Method validation

The method described above was validated with regard to linearity, sensitivity, accuracy, precision, specificity, and stability according to "The Guidance for Industry Bioanalytical Method Validation" published by the Food and Drug Administration [13].

2.8.1 Linearity and sensitivity

The analytical range to be validated was chosen on the basis of the expected plasma concentrations of the studied ARI and CLO drugs [14, 15]. The calibration curves were done for ARI and CLO in the biological sample. The calibration curves should consist of a blank sample (matrix sample processed without I.S), a zero sample (matrix sample processed with I.S), and six to eight samples covering the expected range, including LLOQ that were prepared by adding the required volume of working solutions of ARI and CLO drugs to blank plasma. The plasma samples were subjected to the sample preparation procedure and injected into the LC. The calibration curves of ARI and CLO were plotted using the peak area ratio of ARI and CLO to the I.S versus the nominal concentration. Six calibration curves models were determined by calculating the linear regressions (correlation coefficient, r).

Sensitivity was defined by the lower limit of quantitation (LLOQ), which was the concentration of ARI and CLO at which the signal to noise (S/N) ratio was greater than 5 with acceptable accuracy and precision. These values were set as the lowest concentrations in calibration curves. The calibration models were accepted if the recoveries were within ± 20% at the lower limit of quantification (LLOQ) and within ± 15% at all other calibration levels and if at least 2/3 of the standards met this criterion, including highest and lowest calibration levels.

2.8.2 Precision and accuracy

The intra- and inter- day precision (expressed as RSD %) and accuracy expressed as (recovery %) were determined by analysis three replicates of each concentration of ARI and CLO on the same day to determine the intra-day accuracy and precision of the method. To confirm the inter-

day precision three replicates of each concentration were analyzed at three separate days.

2.8.3 Specificity

Specificity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample [13]. The specificity of the method was investigated by analyzing six blank plasma samples. Each blank sample was tested for interference using proposed extraction procedure and the response of the endogenous compounds at the retention times of the studied drugs in plasma samples and by a separation of the two peaks of ARI and CLO with acceptable resolution factor.

2.8.4 Extraction recovery

The recoveries of ARI, CLO and I.S from plasma were evaluated by comparing the mean peak areas of triplicate analyses of three unextracted samples of drugs in methanol to mean peak areas of the same concentrations with prepared spiked plasma samples.

2.8.5 Drug stability in biological fluid

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. The stability of ARI and CLO drugs in human plasma (expressed as recovery % ± RSD) was investigated in four ways:

2.8.5.1 Short term stability (STS)

Three aliquots of each of the low, medium and high concentrations should be thawed at room temperature and kept at this temperature for 4 hours (based on the expected duration that samples will be maintained at room temperature in the study) and analyzed.

2.8.5.2 Post-preparative stability (PPS)

The stability of processed samples, including the resident time in the autosampler, should be determined. In this study, three replicates were prepared, and kept at room temperature for approximately 24 h.

2.8.5.3 Freeze-thaw stability (FTS)

Three aliquots at each of the low, mid and high concentrations were stored at (-20°C) for 24 h and thawed at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze–thaw cycle were repeated three times, and then analyzed.

2.8.5.4 Long-term stability (LTS)

Long-term stability was determined by storing five aliquots of each of low, mid and high concentrations of the studied drugs at -20°C for 30 days.

The concentrations of all the stability samples were compared to the mean values for the standards at the appropriate concentrations (low, medium and high samples) from the first day of long-term stability testing.

3 Results and Discussion

An accurate, sensitive, selective and validated HPLC method was developed for simultaneous determination of ARI and a common co-administered mood stabilizer drug; Clonazepam in human plasma.

3.1 Method optimization

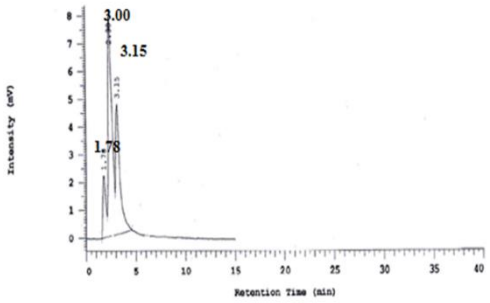

Fig.3. Chromatogram of blank plasma

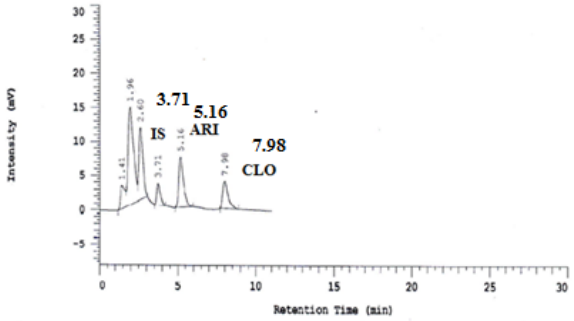

Fig.4. Chromatogram of extracted ARI, CLO and IS from human plasma.

The chromatographic conditions, especially the composition of mobile phase, were optimized to achieve a good resolution and symmetric peak shapes for the two drugs and the internal standard, as well as a short analytical time. Initially a mixture of acetonitrile: 0.05 M potassium dihydrogen orthophosphate, pH=3.6 was used as a mobile phase with a ratio of (60: 40 v/v). The resolution between the peaks of internal standard and ARI was less than 1.5 and the I.S peak was attached to plasma peak. Increasing the buffer ratio (polarity increase) to 60% resulted in increasing the retention of I.S and ARI slightly away from the plasma peak. The mobile phase used for the simultaneous determination of ARI and CLO was mixture of acetonitrile and potassium dihydrogen orthophosphate, pH (3.6) in a ratio of (40: 60 v/v). The chromatograms for the plain plasma and the mixture of ARI (t_R= 5.16), CLO (t_R= 7.98) and I.S (t_R= 3.71) are shown in **Fig. 3 and 4**, respectively. Upon utilizing the above conditions for the

determination of ARI and CLO in six different plasma sources, the absolute peak overcome the matrix effect and gave reproducible results. Calibration graphs were obtained by plotting the peak area ratio of ARI and CLO to that of the I.S versus the nominal concentration.

Linearity range was found to be 20-1000 ng mL^{-1} using the following regression equations:

For ARI: A =0.0009 C + 0.0592 r = 0.9995

For CLO: A =0.020 C – 0.0593 r = 0.9996

Where, A represents the peak area ratio, C is the concentration in ng mL^{-1} and r is the correlation coefficient.

3.2 Method validation

3.2.1 Linearity and lower limit of quantification (LLOQ)

Calibration curves were linear in the concentration range of 20-1000 ng mL^{-1} for ARI and CLO. The validation parameters are listed in **Table 1**. The coefficient of determination (r^2) of the calibration curves was ≥ 0.999. The lowest limit of quantification for ARI and CLO was determined to be 20 ng mL^{-1}, with a signal to noise ratio (S/N) of 5.5.

Table 1. Validation parameters for the proposed HPLC method, for determination of ARI and CLO in spiked human plasma.

Validation Parameters	ARI	CLO
Linearity range (ng mL^{-1})	20-1000	20-1000
Correlation coefficient (r)	0.9995	0.9996
Slope	0.0009 ± 0.018	0.020 ± 0.012
Standard error of the slope	1.265×10^{-5}	26.06×10^{-5}
Intercept	0.0592	-0.0593
Standard error of the intercept	0.007113	0.1465
Standard deviation of residuals from line	0.01146	0.2360
LLOQ (ng mL^{-1})	20.0	20.0

[a] Average of six separate determinations.

3.2.2 Precision and accuracy

The intra- and inter-day precision and accuracy of five concentrations of ARI and CLO drugs are summarized in Table 2. For ARI, The intra- and inter-day RSDs were less than 2.82 and 2.94, respectively. The intra-day accuracy ranged from 87.43 to 97.48 %. For CLO, The intra- and inter-day RSDs were less than 2.31and 1.89, respectively. The intra-day accuracy ranged from to 89.64 to 97.54 %. The above values show that the method is accurate and precise.

3.2.3 Specificity

Specificity was assessed to show that the intended analytes are measured and that their quantitation is not affected by the presence of the biological matrix. Retention times of ARI, CLO and the IS were 5.16 ± 0.10 min, 7.98 ± 0.12 min and 3.71 ± 0.12 min, respectively. No significant interference from endogenous peaks was observed at these retention times.

3.2.4. Extraction recovery

Absolute recovery was calculated by comparing peak areas obtained from freshly prepared sample extracted with unextracted standard solutions of the same concentration. Recovery data was determined in triplicates at three concentrations (low, medium, high) as recommended by the FDA guidelines 13. The good recovery of ARI and CLO from plasma using the (acetonitrile: methanol) mixture proved that this extraction method reliably eliminated interfering material from plasma. The mean percent recovery values of ARI and CLO at low, medium and high quality control levels are listed in Table 3. While the mean percent recovery of the IS at a concentration of 500 ng mL-1 was 91.6 with an acceptable precision (RSD < 8%).

3.2.5 Drug stability in biological fluid

The stability of ARI and CLO drugs under various conditions is shown in **Table 4**. All results were within the acceptance criteria of ± 15% deviation from the nominal concentration.

Table 2. Precision and accuracy of the proposed HPLC method for determination of ARI and CLO in spiked human plasma

	ARI		Accuracy	CLO		Accuracy
	Taken conc. (ng mL^{-1})	**RSD (n=5)**	**Recovery % [a]**	**Taken conc. (ng mL^{-1})**	**RSD (n=5)**	**Recovery % [a]**
Intra-day precision	50	2.82	87.43	50	0.67	89.64
	200	0. 74	93.65	200	1.36	90.58
	500	1.46	96.32	500	1.02	94.00
	700	1.17	97.48	700	0.97	95.37
	900	1.35	95.14	900	2.31	97.54
	Taken conc. (ng mL^{-1})	**RSD (n=5)**		**Taken conc. (ng mL^{-1})**	**RSD (n=5)**	
Inter-day precision	50	2.45		50	1.32	
	200	2.13		200	0.36	
	500	2.66		500	0.42	
	700	1.87		700	1.21	
	900	2.94		900	1.89	

[a] Average of three separate determinations.

Table 3. Results of recovery of ARI and CLO in human plasma, by the proposed extraction procedure

Concentration (ng mL^{-1})	ARI		CLO	
	Recovery [a]	**RSD [a]**	**Recovery [a]**	**RSD [a]**
20	89.43	4.43	86.36	2.86
400	91.82	3.12	88.38	5.76
800	90.76	4.16	88.74	4.28

[a] Average of three separate determinations.

Table 4. Stability study of ARI and CLO in human plasma by the proposed HPLC method

Stability parameters	ARI		CLO	
	Plasma conc. (ng mL^{-1})	Recovery % ± RSD [a]	Plasma conc. (ng mL^{-1})	Recovery % ± RSD [a]
Short term (4 h, room temperature)	20	95.87 ± 2.54	20	98.43 ± 0.87
	400	97.32 ± 2.76	400	96.52 ± 1.55
	800	93.21 ± 2.21	800	96.32 ± 1.81
Post-preparative (24 h, room temperature)	20	94.32± 3.86	20	97.51 ± 1.96
	400	92.43 ± 3.53	400	94.85 ± 3.15
	800	92.38 ± 4.43	800	95.71 ± 2.87
Freeze – thaw (three cycles)	20	88.30 ± 2.45	20	95.63 ± 3.21
	400	91.75 ± 2.03	400	97.92 ± 1.47
	800	89.58 ± 1.67	800	94.22 ± 2.85
Long term (– 20°C, 30 days)	20	87.74 ± 2.87	20	92.65 ± 2.22
	400	87.53 ± 4.36	400	90.84 ± 2.85
	800	90.75 ± 4.85	800	88.73 ± 4.23

[a] Average of three separate determinations.

4 Conclusion

A sensitive, accurate, precise and validated HPLC method was developed for simultaneous determination of ARI and co-administered clonazepam drug in human plasma. The method is economical and fast (simple extraction procedure) and it could be used in the future bioequivalence studies and as an alternative procedure for routine therapeutic drug monitoring of patients treated with Aripiprazole and Clonazepam combination.

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