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RESEARCH ARTICLE

A Novel Method for The Simultaneous Determination of Olanzapine and Escitalopram in Artificial Saliva by High Performance Liquid Chromatography

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SUMMARY

Monitoring of the drug levels can be very important for efficient drug treatment. For this purpose, studies on drug analysis from various biological fluids (especially plasma) are carried out in analytical chemistry. For many drugs, determination of drug concentration in saliva can be used alternatively for drug level monitoring. Patients with cognitive dysfunction have difficulty in maintaining usual living standards and adapting to society. Individuals with such disorders need to be treated with an appropriate medication regimen. Generally, treatment is provided orally and the most commonly used antipsychotic drugs are olanzapine (OLZ) and escitalopram (ESC). Measurement of saliva drug concentration of OLZ and ESC can be helpful for the treatment of diseases. In this study, it is aimed at developing a novel HPLC method that will allow OLZ and ESC to simultaneously be determined in artificial saliva. The separation was achieved on XBridge, C18 column with diode array detector (DAD) (240 nm) and isocratic elution of mobile phase containing acetonitrile and phosphate buffer mixture (20 mM NaH_2PO_4, pH 4.6) (35:65, v/v) containing mobile phase at a flow rate of 0.9 mL/min. Drug extraction from artificial saliva was applied using a methanol and acetonitrile (1:1; v/v) mixture. The recoveries were found in the range of 97.508% and 104.49% (mean) for OLZ and ESC, respectively, from artificial saliva.

Key Words: *Olanzapine, Escitalopram, Saliva, HPLC, Validation.*

Olanzapin ve Essitalopramın Yapay Tükürükte Yüksek Performanslı Sıvı Kromatografisi ile Eşzamanlı Tayini için Yeni Bir Yöntem

ÖZ

İlaç seviyelerinin izlenmesi, etkili ilaç tedavisi için çok önemli olabilir. Bu amaçla analitik kimyada başta plazma olmak üzere çeşitli biyolojik sıvılardan ilaç analizine yönelik çalışmalar yürütülmektedir. Birçok ilaç için tükürük, ilaç konsantrasyonu tayini, ilaç seviyesi takibi için alternatif olarak kullanılabilir. Bilişsel işlev bozukluğu olan hastalar normal yaşam standartlarını sürdürmekte ve topluma uyum sağlamakta zorluk çekerler. Bu tür bozuklukları olan bireylerin uygun bir ilaç rejimi ile tedavi edilmesi gerekir. Genellikle tedavi ağız yoluyla sağlanır ve en yaygın kullanılan antipsikotik ilaçlar olanzapin (OLZ) ve essitalopramdır (ESC). OLZ ve ESC'nin tükürük ilaç konsantrasyonunun ölçülmesi hastalıkların tedavisi için yararlı olabilir. Bu çalışmada, OLZ ve ESC'nin yapay tükürükte eş zamanlı olarak belirlenmesini sağlayacak yeni bir HPLC yönteminin geliştirilmesi amaçlanmıştır. Ayırma işlemi XBridge, C18 kolonunda diyot dizi dedektör (DAD) (240 nm) ve asetonitril ve fosfat tampon karışımı (20 mM NaH_2PO_4, pH 4.6) 35:65 (h/h) içeren mobil fazın izokratik elüsyonu ile 0,9 mL/dak akış hızında gerçekleştirilmiştir. Yapay tükürükten ilaç ekstraksiyonu, metanol ve asetonitril (1:1; v/v) karışımı kullanılarak uygulanmıştır. Yapay tükürükten geri kazanım değerleri OLZ ve ESC için sırasıyla %97.508 ve %104.49 (ortalama) aralığında bulunmuştur.

Anahtar Kelimeler: *Olanzapin, Essitalopram, Tükürük, HPLC, Validasyon*

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INTRODUCTION

Cognitive dysfunction is one of the most critical health problems that reduce the quality of life and can affect both the individual and their environment. Patients with cognitive dysfunction (psychotic disorder) have difficulties living as healthy and adapting to society, if they cannot access appropriate treatment, the quality of life of the patient and the patient's environment decrease. The most essential treatment method for psychotic disorders is the use of antipsychotic drugs. The two most commonly used drugs are olanzapine (OLZ) and escitalopram (ESC).

OLZ is an atypical antipsychotic drug approved by the US Food and Drug Administration (FDA) for the treatment of schizophrenia, and bipolar disorder. It is also recommended for patients with anorexia nervosa, autism spectrum disorders, Tourette syndrome and tic disorders. OLZ is also used to treat resistant depression and depressive episodes in bipolar disorder (Dziurkowska et al., 2020). Molecular formula is $C_{17}H_{20}N_4S$ and chemically named 2-methyl-4-(4-methylpiperazin-1-yl)-10*H*-thieno[2,3-b][1,5]benzodiazepine (Figure 1).

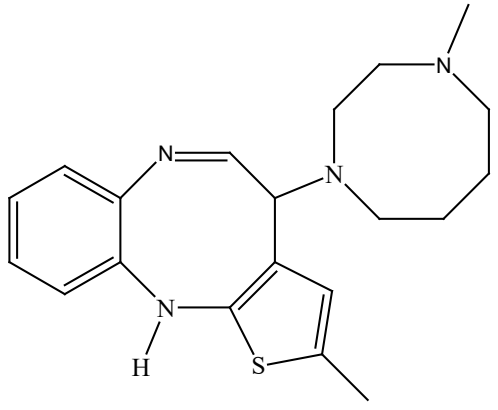

Figure 1. Chemical structure of olanzapine

ESC is a widely used antidepressant approved by the US Food and Drug Administration (FDA) in 2001 and prescribed to more than 240 million patients to date. ESC is an oral serotonin reuptake inhibitor (SSRI). It is effective in the treatment of moderate to severe generalized anxiety disorder, panic attacks (agoraphobia), and obsessive-compulsive disorder and is generally well tolerated (Garnock-Jones et al., 2010). Molecular formula is $C_{20}H_{21}FN_2O$ and chemically named (1*S*)-1-[3-(dimethylamino) propyl]-1-(4-fluorophenyl)-3H-2-benzofuran-5-carbonitrile (Figure 2).

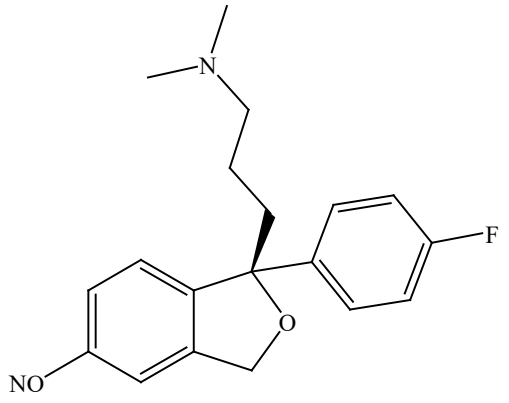

Figure 2. Chemical structure of escitalopram

Determination of adequate drug levels of antipsychotic drugs plays a very important role in the course and treatment of the diseases. For this reason, developing methods for drug analysis in various biological fluids is one of the most important fields of study in analytical chemistry. Analytical methods for the determination of antipsychotic drugs need to be highly sensitive and selective for accurate and precise quantification.

High-performance liquid chromatography (HPLC) methods are generally preferred for determining of drug levels in plasma or other biological materials as they provide sensitive and accurate results.

With the methods developed with HPLC, it is possible to reach the analysis result in a very short time. In this study, it is aimed to develop a validated HPLC method that allows simultaneous analysis of OLZ and ESC in artificial saliva.

Few studies have been published in the literature for the simultaneous determining of ESC and OLZ. The method developed by Alasabashi et al. did not include stability studies, and the analysis of ESC and OLZ in

biological fluids was not performed (Alasabashi et al., 2022). A method for the simultaneous analysis of OLZ and ESC in saliva by HPLC is not yet available.

In the studies conducted, OLZ and ESC are usually quantified in blood. Monitoring serum and plasma levels to determine drug concentrations and monitor patient treatment is still one of the most commonly used methods in routine controls. However, drug determination methods in serum and plasma can pose many problems in practice due to various difficulties in the collection of diagnostic material (Chiu & Franklin 1996; Raggi et. al., 2000; Boulton et al., 2001; Bhimanadhuni et al., 2012; Teichert et al., 2020). For example, drawing blood from a patient can have adverse effects such as fear, pain, skin irritation, and infection. In addition, professional medical personnel are required to obtain serum or plasma for analytical research. For this reason, alternative sample options are being developed to reduce the amount of work and risk involved.

The advantage of choosing saliva as the matrix, which can be an alternative to plasma and serum, is related to the non-invasive nature of the collection procedure, which requires fewer safety precautions must be taken and the health professional gathering the sample needs to possess less training. The amount of drugs detected in saliva is reported to reflect the concentration of a free, non-protein-bound drug in plasma. Given that only the free form of the drug exerts pharmacologic activity, the importance of saliva analysis in pharmacokinetic studies is evident. However, further research is needed before saliva analysis can replace plasma analysis for each drug (Abad 1983). For these reasons, this study aimed to develop a sensitive, accurate, and rapid HPLC method to determine the levels of OLZ and ESC from artificial saliva, an easily accessible diagnostic material.

MATERIAL AND METHOD

Chemicals and reagents

Analytical standards of OLZ, % purity 99.8, and ESC, % purity 99.7 were obtained from Ali Raif İlaç Sanayi A.Ş., Quercetin (Q; internal standard, IS) was purchased from Merck, (Darmstadt, Germany). Acetonitrile (HPLC grade, CARLO ERBA, Italy), methanol (HPLC grade, CARLO ERBA, Italy), NaH_2PO_4, KCl, NaCl, NaOH, $CaCl_2$, KSCN, NH_2CONH_2, and Na_2S were purchased from Sigma-Aldrich (USA). OLZ, ESC and Q stock solutions were prepared with methanol at 1 mg/mL. The solutions to be used in the analysis were prepared daily by diluting the stock solutions with methanol to the desired concentration values. Stock solutions were stored at -20 °C for 1 week.

Instrumentation and chromatographic conditions

The chromatographic system consisted of a Shimadzu liquid chromatograph equipped with a pump (LC-10AT VP), a controller (SCL-10A VP) connected to a computer using software (Class-VP 5.03), an autosampler (SIL-10AD VP), 10 µL injection loop and Diode Array Detector (DAD, SPD-10A VP). The system was controlled through a system controller (SCL-10A), a personal computer using a CLASS-VP 5.0 workstation with a data processing system (Shimadzu, Kyoto, Japan) installed on it. The separation was performed on a XTerra, C_{18} (100 x 4.6 mm i.d., 3.5 µm), and XBridge, C_{18} (250 x 4.6 mm, particle size 5 µm) analytical columns (Waters, Milford, MA, USA). The column temperature was set to 24 °C. The mobile phase consisted of 20 mM NaH_2PO_4 (pH adjusted to 4.6 with NaOH), and acetonitrile in the ratio of 35:65 (v/v) in isocratic mode and the DAD detector was set to the wavelength of 240 nm. 10 µL of sample solutions was injected into the HPLC system at the flow rate of 0.9 mL/min. Quercetin was chosen as the internal standard as its peak was very well resolved from two drug peaks, showing any interfering peaks. All solutions were prepared in type 1 water (Simplicity 185 Water System, Millipore Corp., Bedford, MA, USA). The mobile phase was filtered through a membrane filter with a pore diameter of 0.45 µm and kept in an ultrasonic bath for 15 min to remove the soluble gases. At end of the analysis, column was flushed with approximately

20 times of column volume of a mixture with HPLC grade water and methanol (50:50, v/v). Finally, the column was stored in pure methanol. This procedure was applied for every analysis.

Drug analysis in artificial saliva

The development of the method of simultaneous analysis of OLZ and ESC in artificial saliva by HPLC was carried out using the standard addition method of active substance components into artificial saliva. For this purpose, artificial saliva was prepared by dissolving the components given in Table 1 in 1 L of pure water (J Pytko-Polonczyk et al; 2017).

Artificial saliva was used to prepare solutions of OLZ and ESC at the desired concentration values, and Q was spiked to the concentration value of 0.5 µg/mL. 1 mL of a solution that was prepared with artificial saliva was taken in a 2 mL Eppendorf tube, then 0.5 mL of a mixture of methanol and acetonitrile (1:1; v/v) was added to the solutions, and then the solutions were mixed with a vortex mixer for 15 s and then centrifuged at 6000 rpm for 6 min. After centrifugation, approximately 300 µL aliquot was taken from the top of the tube without shaking. Aliquot was filtrated by a 0.45 µm nylon injector filter, and then 10 µL of a solution was injected into the HPLC system. This procedure was repeated two times for each sample. The developed method was validated according to International Conference on Harmonization (ICH) requirements (Validation of Analytical Procedures: Text and Methodology Q2 (R1), 2014; Validation of Analytical Procedures Q2(R2), 2022).

Table 1. Artificial saliva content (for 1 L, pH=6.5)

Compound	Quantity (mg)
KCl	400
NaCl	400
$NaH_2PO_4.H_2O$	690
$CaCl_2.2H_2O$	795
$Na_2S.9H_2O$	5
KSCN	300
CH_4N_2O	1000

Method validation

Validation studies were performed according to ICH requirements. Within the scope of the validation studies carried out, retention time, capacity factor, tailing factor, and theoretical number of plates for each drug were calculated. The system suitability test was performed with six repeated injections of the standard mixture solution at a concentration of 1 µg/mL. In the method, the calibration curve was obtained by the peak area under the analyte peak being divided by the peak area of the IS peak area, and the obtained values were plotted against the concentration value of each drug. The method linearity was evaluated by examining the correlation coefficient (r) values from the obtained curves. The linearity was calculated using the regression equation (y = mx + n) data, including concentration ranges, correlation coefficients, and the standard error of intercept. In addition, sensitivity, selectivity, accuracy, recovery and precision studies were performed at three different concentration values (1, 2 and 3 µg/mL, n=6) and intra-day and inter-day repeated analysis results were evaluated. The limit of detection (LOD) value was taken as the signal-to-noise (S/N) value equal to 3 for repeated (n=6) different standard solution analyses. The limit of quantification (LOQ) value was taken as the value where the RSD value was less than or equal to 5% for repeated (n=6) different solution analyses and the signal-to-noise (S/N) value equal to 10 for repeated (n=6) different standard solution analyses (Jenke 1996; Jenke 1996).

RESULTS AND DISCUSSION

To determine the optimum chromatographic separation conditions, various mobile phase compositions and different stationary phases were tested. For this purpose, the effect of the ratio of organic composition as acetonitrile and methanol in the mobile phase was investigated at different concentrations and different elution applications. For this purpose, XTerra, C_{18} (250 x 4.6 mm, particle size 3.5 µm) and XBridge, C_{18} (250 x 4.6 mm, particle size

5 µm) analytical columns were tested as stationary phase and the best separation was achieved with XBridge, C_{18} column. Again, the optimization studies of the mobile phase composition were carried out with buffer solutions at different pH values; the effects of pH values between 3.5 and 5.0 on the capacity factor and peak symmetry ratios for separation were examined. Also, the concentration of buffer solution was investigated according to the capacity factor. The best separation was obtained for OLZ, ESC, and Q in isocratic elution with the mobile phase composition of acetonitrile and phosphate buffer (20 mM, pH 4.6) 35:65 (v/v) at a flow rate of 0.9 mL/min (24°C).

For validation studies, specificity, LOD, LOQ, selectivity, linearity, accuracy, sensitivity, intra-day and inter-day precision were investigated for developed method.

The method was linear in the range of 0.8-3 µg/mL for OLZ and ESC. The r^2 (regression coefficient) values from the curve equations obtained were close to 1 (Table 2). The LOD and LOQ values for OLZ and ESC in artificial saliva were to be 0.3 µg/mL and 0.8 µg/mL, respectively.

Table 2. Calibration curve parameters

Drug	Slope	Intercept	r^2	Slope SE*	Intercept SE*	LOD (µg/mL)
OLZ	2.0151	0.5992	0.9981	0.084	0.093	0.3
ESC	1.4639	0.603	0.9914	0.079	0.078	0.3

SE*: Standard Error (n=6)

As a result of the studies carried out to determine the selectivity of the method, it was determined that there was no interference peak in the retention times of OLZ, ESC, and IS peaks caused by artificial saliva, mobile phase, or HPLC system (Figure 3).

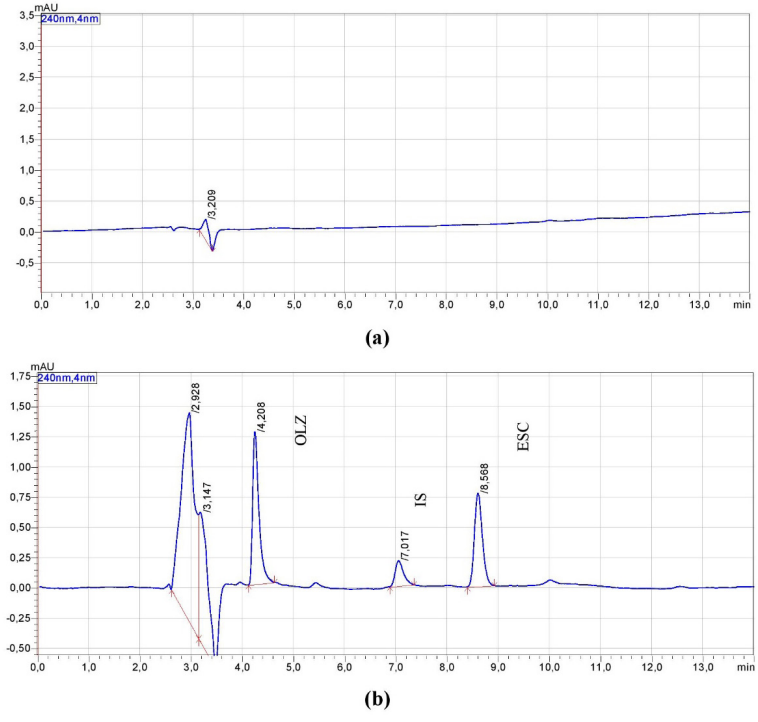

Figure 3. (a) Chromatogram of artificial saliva without drugs, (b) Chromatogram of artificial saliva extraction containing 1.5 µg/mL OLZ and ESC

Suitability parameters including retention time, capacity factor, tailing factor, theoretical number of plates were investigated (Table 3).

HPLC-DAD system suitability for the developed method was evaluated based on retention time, injection repeatability, capacity factor, tailing factor and the theoretical number of plates. The data obtained from 6 replicate experiments were used for the investigated parameters. The values obtained (Table 3) meet the accepted conditions of capacity factor k values for good separations, tailing factor≤1.5, and the theoretical number of plates>2000, and the system was found to be suitable for analyzing targeted drugs. Calculated % RSD values of retention time and capacity factor, tailing factor parameters were also determined (Jenke 1996; Jenke 1996).

Table 3. System suitability parameters

Active ingredients	Retention time* (min)	Capacity factor, k*	Tailing factor*	The Theoretical number of plates
OLZ	4.25 ± 0.21	0.34 ± 0.07	1.65 ± 0.17	2742.97
ESC	8.52 ± 0.05	1.66 ± 0.02	1.21 ± 0.04	24545.31
IS	7.01 ± 0.11	1.19 ± 0.04	1.46 ± 0.16	9618.06

*n=6, results are given by mean ± relative standard deviation

Within the precision studies, intra-day, inter-day repeated (n=6) analyses were performed at concentrations of 1, 2 and 3 µg/mL. In the extraction studies, the effects of methanol, acetonitrile and extraction solutions individually or in different mixture combinations on the analysis were examined. The best extraction solvent mixture was selected as a mixture of methanol and acetonitrile (1:1; v/v). The data obtained after extraction were analyzed by calculating the mean, relative standard deviation, and standard deviation values (Table 4).

Table 4. Inter-day and intra-day precision and accuracy results (n=6)

Added (µg/mL)	Parameters	OLZ		ESC	
		Inter-day	Intra-day	Inter-day	Intra-day
1	Found	0.981	0.979	1.027	1.045
	Precision RSD (%)	1.282	0.430	0.982	1.363
	RE	-0.509	-0.487	-0.511	-0.478
	Accuracy Bias (%)	-1.865	-2.130	2.684	4.490
	Recovery (%)	98.135	97.870	102.684	104.490
2	Found	2.051	2.016	2.047	2.056
	Precision RSD (%)	1.370	0.912	0.256	0.205
	RE	-0.487	-0.496	-0.488	-0.486
	Accuracy Bias (%)	2.536	0.815	2.331	2.802
	Recovery (%)	102.536	100.815	102.331	102.802
3	Found	2.925	3.069	3.053	3.288
	Precision RSD (%)	2.495	0.985	2.401	2.343
	RE	-0.483	-0.483	-0.490	-0.388
	Accuracy Bias (%)	-2.492	1.778	3.339	2.019
	Recovery (%)	97.508	101.778	103.33	102.019

$\bar{x}$: Mean value, RE: Relative error, RSD**:** Relative Standard Deviation,

Bias**:** ((amount found - amount added)/amount found) x100

The studies carried out to investigate the recovery values of OLZ and ESC from artificial saliva (n=6), from the data obtained, the recovery values were found in the range of 97.508% and 104.49%.

It is crucial to establish a method for choosing saliva as an alternative for analyzing serum and plasma levels to ascertain drug concentrations and for patient therapy. Saliva sample collection is more accessible to implement as it requires fewer safety precautions and is a non-invasive sampling procedure. The HPLC method developed for OLZ and ESC analysis in artificial saliva is presented as an easily applicable method when the recovery and accuracy parameters are examined. The developed method is sensitive, with a LOD of 0.3 µg/mL. It has short retention times with a separation time of approximately 10 minutes. In addition, proposed practical extraction procedure for drug determination can be helpful further analysis procedures. The developed method is superior to the existing methods in the literature regarding precision and easy applicability.

CONCLUSION

Analyzing of drugs in biological material is critical in determining an effective treatment regimen. In this study, a new method for OLZ and ESC analysis in artificial saliva was developed. Due to ethical limitations, the study could not be performed in patient saliva samples. OLZ and ESC analysis in blood and saliva samples from patients may give more precise results in terms of the usability of saliva in determining drug levels. In addition, this can be said in light of previous studies on the usability of saliva as a substitute for plasma drug levels. In this study, a novel easy, simple, selective and validated chromatographic method was developed for OLZ and ESC in artificial saliva allowing simultaneous analysis in HPLC-DAD system. The developed method may be necessary as it has not been done in the literature and can be presented as a new and applicable approach and method in drug treatment processes. The developed analysis method can be applied as a new, simple method in drug-level monitoring and different applications.

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AUTHOR CONTRIBUTION STATEMENT

Idea (EDGT, AD), planning and method development of HPLC analyses (AD), the manuscript designing and editing (AD), performing experiments (AD, SŞ), investigation and literature review (AD, SŞ, EDGT).

CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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