J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 48(1): 189-196, 2024
Doi: 10.33483/jfpau.1345130

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DEVELOPMENT AND VALIDATION OF A NEW UHPLC-DAD APPROACH FOR ATOMOXETINE DETECTION IN SEVERAL MEDICINAL PLANTS

BAZI TIBBİ BİTKİLERDE ATOMOKSETİN TAYİNİ İÇİN YENİ BİR UHPLC-DAD YÖNTEMİNİN GELİŞTİRİLMESİ VE VALİDASYONU

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ABSTRACT

Objective: *Atomoxetine (ATX) is a medication that is extensively used to treat attention deficit hyperactivity disorder in children, adolescents, and adults. The goal of this work was to create a speedy, easy, and sensitive ultra high performance liquid chromatographic method (UHPLC) for the measurement of atomoxetine in various medicinal plants. (Salvia officinalis L., Rosmarinus officinalis L., Melissa officinalis L., Ginkgo biloba L.).*
Material and Method: *Prior to chromatographic separation, liquid-liquid extraction was applied, which is currently the preferred extraction technique due to its simple, fast and efficient procedure for sample preparation. The chromatographic separation was achieved by reversed phase C18 (5 µm × 4.6 mm × 150 mm) analytical column and a mobile phase consisting of monobasic potassium dihydrogen orthophosphate (pH=6.8) and acetonitrile (50:50 v/v) at flow rate of 0.8 ml/min and diode array detector (DAD) detecting at 215±2 nm.*
Result and Discussion: *The envisioned method's linear behavior was tested in the 0.5-20 µg/ml range (r^2=0.09990). In compliance with International Conference on Harmonisation (ICH) criteria, the method received validation by means of accuracy, precision, repeatability, specificity, robustness, and detection and quantification boundaries. LOD and LOQ values were determined as 0.16 and 0.5 µg/ml. RSD values for hourly and daily measurements are found to be below 2.5% for both assays. The proposed method can be used effectively for quantification of atomoxetine in medicinal and aromatic plants. The proposed analytical procedure represents an efficient method for the quantification and routinee analysis of atomoxetine in medicinal and aromatic plants.*
Keywords: *Atomoxetine, attention deficit hyperactivity disorder, diode array detector (DAD), medicinal plants, ultra high performance liquid chromatography (UHPLC)*

ÖZ

Amaç: *Atomoksetin (ATX) çocuklarda, ergenlerde ve yetişkinlerde dikkat eksikliği hiperaktivite bozukluğunun tedavisinde yaygın olarak kullanılmaktadır. Bu çalışmada, bazı tıbbi bitkilerde (Salvia officinalis L., Rosmarinus officinalis L., Melissa officinalis L., Ginkgo biloba L.) atomoksetin analizi için hızlı, basit ve hassas bir ultra yüksek performanslı sıvı kromatografik yöntem (UHPLC) geliştirilmesi amaçlanmıştır.*
Gereç ve Yöntem: *Kromatografik ayırmadan önce, numune hazırlama için basit, hızlı ve verimli prosedürü nedeniyle günümüzde tercih edilen ekstraksiyon tekniği olan sıvı-sıvı ekstraksiyonu uygulanmıştır. Kromatografik ayırma, ters fazlı C18 (5 µm × 4.6 mm × 150 mm) analitik kolon ve*

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Submitted / Gönderilme : 17.08.2023
Accepted / Kabul : 18.12.2023
Published / Yayınlanma : 20.01.2024

monobazik potasyum dihidrojen ortofosfat (pH=6.8) ve asetonitrilden (50:50 h/h) oluşan bir mobil fazda 0.8 ml/dk akış hızında ve 215±2 nm'de diyot dizisi dedektörü (DAD) tespiti ile sağlandı.
Sonuç ve Tartışma: *Önerilen yöntemin doğrusallığı 0.5-20 µg/ml (r^2=0.9990) aralığında incelenmiştir. Yöntem, Uluslararası Uyumlaştırma Konferansı (ICH) yönergelerine uygun olarak doğruluk, kesinlik, tekrarlanabilirlik, özgüllük, sağlamlık ve dedeksiyon ve kantitasyon limitleri açısından doğrulanmıştır. LOD ve LOQ sırasıyla 0.16 ve 0.5 µg/ml olarak bulundu. Gün içi ve günler arası RSD değerleri her iki test için de %2.5'in altındadır. Önerilen yöntem, tıbbi ve aromatik bitkilerde atomoksetin miktarının belirlenmesi için etkin bir şekilde kullanılabilir. Önerilen analitik prosedür, tıbbi ve aromatik bitkilerde atomoksetinin miktarının belirlenmesi ve rutin analizi için etkili bir yöntemi temsil eder.*
Anahtar Kelimeler: *Atomoksetin, dikkat eksikliği hiperaktivite bozukluğu, diyot dizisi dedektörü (DAD), tıbbi bitkiler, ultra yüksek performanslı sıvı kromatografisi (UHPLC)*

INTRODUCTION

The condition that is most frequently identified and treated in children is attention-deficit hyperactivity disorder (ADHD), which affects 5-12% of children and adolescents globally. It has been linked with significant morbidity and worse results later in life [1,2]. The first non-stimulant oral selective norepinephrine reuptake inhibitor is atomoxetine hydrochloride (ATX) [(-)-N-methyl-3-phenyl-3-(o-tolyloxy)-propylamine hydrochloride] shown in first figure. In November 2002, the US Food and Drug Administration legally adviced it positively as a usable drug ingredient for ADHD in people aged 6 and up [3,4].

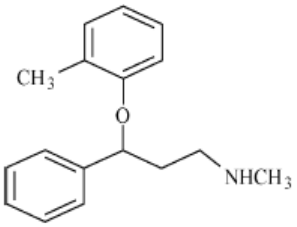

Figure 1. Chemical structure of ATX

There is no known precise mechanism through which ATX exerts its therapeutic effects in ADHD. The ATX has minimal affinity for other neural transporters or neurotransmitter receptor sites and increases norepinephrine function by blocking the presynaptic norepinephrine transporter in a highly selective manner. This retains a larger level of norepinephrine active in the brain's inter-neuron junctions [5-7]. The clearance of ATX displayed a bimodal distribution in studies with this molecule in healthy human volunteers, indicating that an enzyme with a genetic variation was involved in the metabolism of ATX [8].

Many ailments have been attempted to be treated with plants throughout the history of mankind. According to the World Health Organization (WHO), 80% of the world's population- roughly 4 billion people- first tried using herbal medicines to treat their health issues. Additionally, active compounds with a plant origin make up around 25% of prescription medications in affluent nations (e.g., vimbilastine, reserpine, quinine, aspirin). discovering new applications for medicinal and aromatic plants, rising the need for natural goods; the use of these plants is expanding daily [9].

So far, the reported analytical methods for atomoxetine include GC [10], LC-MS [11], and HPLC-UV [12]. None of these studies included the determination of atomoxetine in medicinal plant extracts using the HPLC-DAD method. In this study, a DAD-detection UHPLC approach was established to analyze ATX at a level of µg/ml in a number of medicinal herbs. Liquid-liquid (LLE) extraction is used as a sample pretreatment process because medicinal plants have a complex matrix. LLE features a quicker and less complicated technique than solid-phase extraction (SPE) and ultrasonic assisted extraction. A straightforward analytical technique, LLE combination to UHPLC, has been devised in the proposed investigation for ATX in several medicinal plants (*Salvia officinalis* L., *Rosmarinus*

officinalis L., *Melissa officinalis* L., *Ginkgo biloba* L.).

MATERIAL AND METHOD

Plant Materials and Chemicals

Plant samples (*Salvia officinalis* L., *Rosmarinus officinalis* L., *Melissa officinalis* L., *Ginkgo biloba* L.) were purchased from local markets (herbalist).

Shanghai Yingxuan Pharmaceutical Science & Technology (China) provided the ATX. Solvents other than plant samples and reagents were analytical quality from Merck (Germany), with the exception of acetonitrile, phosphate buffer, NaOH, and ethanol which were HPLC grade. The water that has been purified using the Milipore Direct-Q system.

Plants and Treatment Solution Preparation

A standard solution of 0.5 to 20 µg/ml of ATX was created by further diluting a stock solution of 200 µg/ml (calculated as free base) of ATX in water. Through the course of the study, the stable stock standard and used solutions were continuously maintained at +4ºC.

1 g of plant materials were incubated in 20 ml solvent at room temperature in a shaking water bath at 100-150 rpm for 3 hours to prepare ethanol extracts. The produced extracts were paper filtered for the filtration process combined 1:1 with the mobile phase for liquid-liquid extraction, and the solution underwent filtering through a 0.45 mm filter prior being measured using UHPLC.

Instrument and Chromatographic Condition

The HPLC studies were performed on an Agilent brand 1260 Infinity mode, HPLC-DAD system for ATX detection. To chromatography, a Phenomenex-C18 (5 µm × 4.6 mm × 150 mm) column was utilized. WTW pH 526 digital pH Meter was used to monitor pH. Various conditions, such as C8 and C18 columns and varying flow rates, were examined in order to determine the best suited approach. To achieve the most efficient chromatographic separation, multiple mobile phase, column type, and stationary phase size combinations were tested at various flow rates and column temperatures.

Validation of the Method

The new approach has been examined using the International Conference on Harmonisation (ICH) guidelines [13].

Linearity and detection and quantification boundaries

The graph used for calibration was constructed using standard samples with concentrations ranging from 0.5 to 20 µg/ml of ATX. The calibration curve for peak area versus ATX concentration was created. Limit of detection (LOD) is the minimum level of a component in a sample analyte which we can detect but can not quantify at a satisfactory level where as the significant variable limit of quantification (LOQ) which is the minimum concentration level that can be measured by the experimental system.

Selectivity of the suggested approach

Selectivity has been referred to the ability of an analysis to be performed correctly considering the presence of the elements that may impact the analysis or be in interference with the substance. These parameters had no effect on the outcome of the analysis throughout the investigation.

Accuracy

For the determination of ATX in plant samples extracts; quality control (QC) samples were prepared in several concentration (2.5, 5.0 and 10 µg/ml) which could be categorized as low, medium and high concentration levels (n=3). The accuracy was indicated by the recovery values and the accuracy of the recovery study was determined by the relative standard deviation (RSD) values of the recovery results in three repeated studies. The amount recovered of the chemical was calculated after the produced plant samples were filtered.

Precision of the method

Precision is used for the ability of an analysis to be performed with the alike samples and/or solvents with identical conditions across various different durations. The precision experiments included hourly and daily examinations.

Robustness of the method

Overall this method is supposed to be robust and it was tested by altering the flow rate, composition, as well as column temperature. The mobile phase proportions were changed from 50:50 (v/v) (acetonitrile-phosphate buffer) to 45:55, however originally the the ratio was 55:45. The flow rate and the column temperature were adjusted to 0.7 and 0.9 ml/min and 25 and 35ºC correspondingly.

Stability

Working standard ATX solutions' stability was examined under various storing settings at three different QC levels. The three storage methods being tested include keeping the samples in autosampler conditions for 24 hours, storing in dark and room temperature for 24 hours, and maintaining in a refrigerator at 4°C for one month. Following are the rates of recovery percentages for the conditions that were tested: 98.7%, 96.5%, and 99.1%, respectively. For all of these demonstrations, the greatest RSD% was 1.27%. It would be accurate to say that ATX was determined to be stable given the entire test settings.

RESULT AND DISCUSSION

Chromatographic Process

It was preferred to employ reversed phase (RP) UHPLC and a use of column (5 µm x 4.6 mm x 150 mm, Phenomenex-C18). Acetonitrile-monobasic potassium dihydrogen orthophosphate (pH=6.8) was put in service as the mobile phase for a flow rate of 0.8 ml/min and an isocratical elution pattern. To get the best chromatogram resolution, the column temperature was fixed at 30ºC. The delay lasts around 1.476±0.004 minutes. In Figure 2 illustrative chromatograms are displayed. Table 1 indicates the chromatographic system suitability parameters.

Table 1. Chromatographic system suitability parameters

Capacity factor*	Resolution*	HETP*	Tailing factor*	Asymmetry factor*	Theoretical plates (N)
7.66	3.30	0.08	1.2	1.1	2420

*Mean values of the parameters of all the points in calibration study are mentioned

Validation of the Analytical Method

Sensitivity and Linearity

By graphing the peak zones of the derivatives with the respective ATX concentrations, linear least-squares regression analysis was used to generate curves for calibration. The value calculated from the calibration curve's equation (n=5) was y=102.8 x - 5.101 (correlation coefficient=0.9990), where y stands for the peak zone of ATX and x represents the ATX concentration.

LOD, limit of detection and LOQ, limit of quantitation were calculated by the use of formulae LOD= 3 SDa/m and LOQ= 10 SDa/m. SDa is the intercept's standard deviation, while b is being the slope. Table 2 provides a summary of the variables necessary for the analytical efficacy of the suggested approach. LOD is 0.16 and LOQ is 0.5 µg/ml.

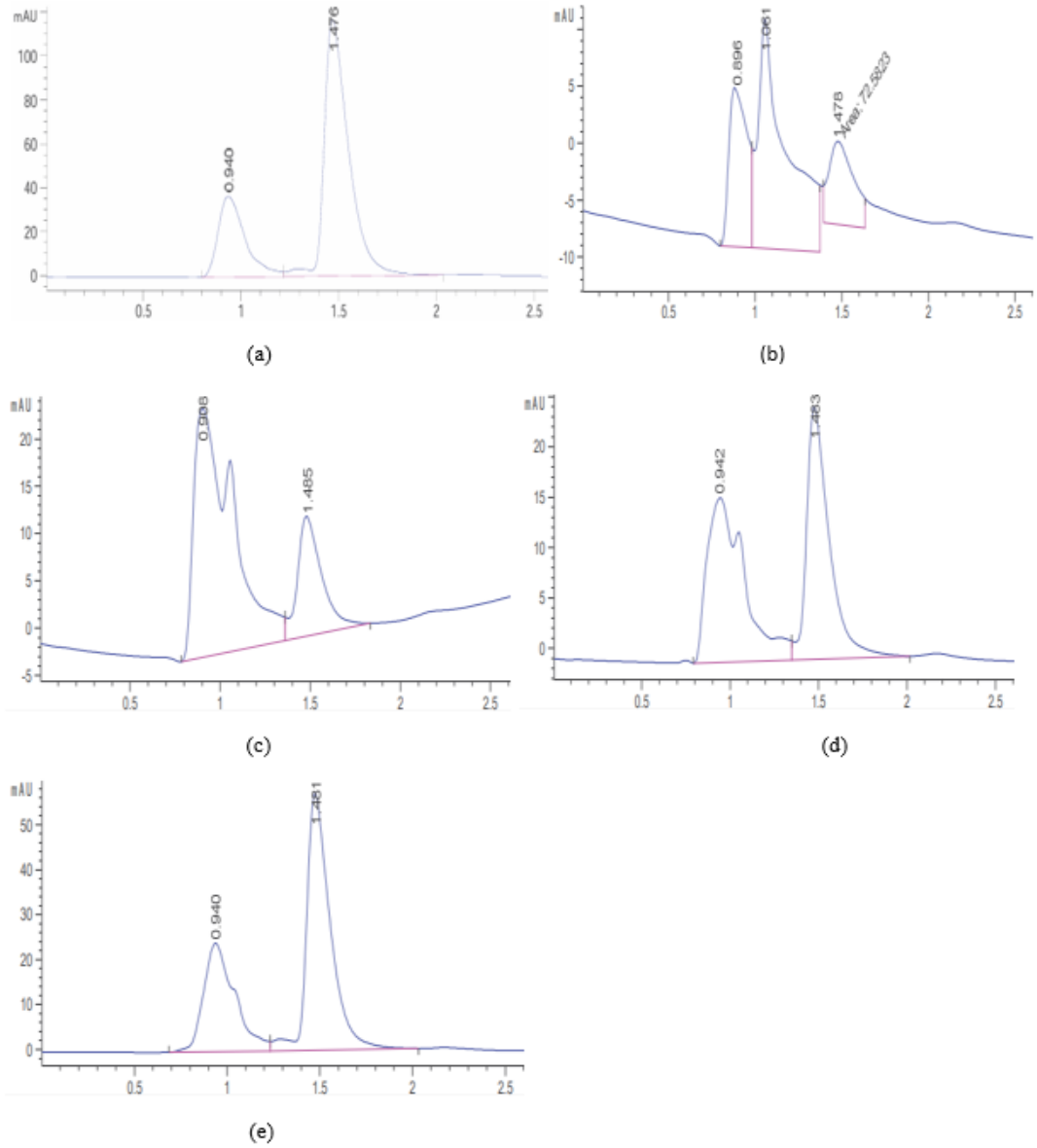

Figure 2. a: standard solution (10 µg/ml standard ATX solution), b: real sample 1 (*Melissa officinalis* L.), c: real sample 2 (*Salvia officinalis* L.), d: real sample 3 (*Rosmarinus officinalis* L.), e: real sample 4 (*Ginkgo biloba* L.)

Table 2. Results for the analytical variables of the suggested approach

Variables	**Results**
Concentration range [a] (µg ml^{-1})	0.5-20.0
Regression equation[b]	y= 102.8x – 5.101
Intercept± SD	5.101 ± 0.457
Slope± SD	102.8 ± 9.22
Correlation coefficient (r^2)	0.9990
LOD (µg ml^{-1})	0.16
LOQ (µg ml^{-1})	0.5

[a] The mean value of three analysis

[b] *C represent contcentrationn in* µg/ml and y represents the peak area for *y=xC* +b

Accuracy and Precision

In order to determine the precision and accuracy values, the samples were examined at three distinct dilution levels. Quality control (QC) specimens were prepared as low, medium, and high concentrations (n=3) at 2.5, 5.0, and 10.0 µg/ml. Standard addition used here for the determination of revocery. Standard addition is a recovery estimation approach. Three identical specimens at each QC concentration in order to be examined for various tests on three consecutive days for daily precision and the same day for hourly precision to display the accuracy of the subjected procedure. Table 3 represents the accuracy and precision results fort he real sample (*Ginkgo biloba* L.) with the highest relative amount of ATX.

Table 3. The outcomes of the precision and accuracy experiments for *Ginkgo biloba* L.

Existant concentration (µg ml^{-1})	**Added concentration (µg ml^{-1})**	**Found concentration (µg ml^{-1}) (Mean±SD[1])**	**Recovery (%)**	**RSD of recovery**	**RSD of intraday variation**	**RSD of interday variation**
10	2.5 5.0 10.0	12.48±0.04 15.01±0.05 20.07±0.06	99.86 100.08 100.35	0.35 0.32 0.27	0.35 0.32 0.27	0.39 0.36 0.33
Mean relative recovery = 100.09						

n=3 for every single concentration

Robustness

By making minor adjustments to the flowrate, column oven temperature, and acetonitrile and water phase concentrations of the mobile phase, robustness was assessed. Temperature column was changed from 30°C to 25°C and 35°C, the mobile phase proportions were modified from (50:50 v/v) (acetonitrile-phosphate buffer) to 45:55 and 55:45; and the flow rate was rised from 0.7 to 0.9 ml/min. Used modifications did not impact the peak areas. Evaluations for robustness are shown in Table 4. The recovery% figures of 104.32, 105.19, and 107.35 are better than those from our earlier study [14].

Table 4. Outcomes of the robustness

Condition	**Value**	**Recovery %**	**RSD %**
Flow rate (ml min^{-1})	0.7 0.9	102.75 104.32	1.76 1.31
Mobile phase composition (ACN:Phosphate buffer)	45:55 55:45	105.19 102.41	2.43 3.21
Column temperature	25 35	107.35 104.63	2.53 4.53

n=3 for each Quality Control samples

The Method's Application to the Identification of ATX from Plant Extracts

After the plant samples were extracted as described in the method section, they were prepared for analysis for UHPLC and the amount of ATX they contained was determined. The relative amounts of ATX in extracts *Salvia officinalis* L., *Rosmarinus officinalis* L., *Melissa officinalis* L., *Ginkgo biloba* L. were determined as 63%, 66%, 42% and 79%, respectively.

Conclusion

Secondary metabolites, unlike primary metabolites, are not directly related to the essential vital activities of the plants. Plants are complex matrices because of the secondary metabolites they contain.

The extraction of target analytes in plant extracts is a difficult process. Liquid liquid extraction is simple and fast compared to other extraction techniques for drug analysis.

Although many medicinal plants are widely preferred by healthcare professionals, patients and the public, no method has been found in the literature to determine ATX in medicinal plants. For the purpose of finding ATX in several medicinal plants, a new sample the development and quantification technique was devised in this work.

The procedure we created is quiet, straightforward, quick, and less expensive. The procedure offers a straightforward mobile phase with isocratic flow and does not call for any derivatization reaction. The developed method can be practically applied in the routine analysis of ATX in herbal extracts, food products and nutraceuticals.

AUTHOR CONTRIBUTIONS

Concept: B.C.; Design: B.C.; Control: B.C.; Sources: B.C.; Materials: B.C.; Data Collection and/or Processing: B.C.; Analysis and/or Interpretation: B.C.; Literature Review: B.C.; Manuscript Writing: B.C.; Critical Review: B.C.; Other:-

CONFLICT OF INTEREST

The author declares that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The author declares that the ethics committee approval is not required for this research.

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