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Prediction of the Trace Amounts of Atomoxetine in Biological Samples using Optimized Solvent Bar Microextraction Technique Coupled with HPLC-UV

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Abstract

Atomoxetine, an attention-deficit/hyperactivity disorder (ADHD) agent, has severe adverse effects, which causes suicidal ideation in patients and when combined with other drugs such as monoamine oxidase inhibitors or in patients with cardiovascular disorders or pheochromocytoma, it can be fatal. In order to monitor the trough level of the drug, sensitive analytical methods are in demand. Therefore, for detection of trace levels of this drug, the trend solvent bar microextraction method coupled with HPLC-UV was implemented. Application of pH gradient of 10.3 and 3.0 (donor and acceptor phase, respectively) enabled preconcentration and microextraction of the target analyte. The results were modeled using Artificial Neural Network (ANN) successfully.

The optimum results were obtained with a stirring rate of 365 rpm and salt addition by 15.1% at 25°C after 26 min. The limit of detection and quantification were 7.0 ng mL^{-1} and 20 ng mL^{-1}, respectively. It offered good linearity range of 20-5000 ng mL^{-1} with a coefficient of estimation higher than 0.9972 and a preconcentration factor of 112. The relative standard deviations of analysis were 4.6% within a day (n=3) and 6.2% between days (n=9).

Overall, this method can be employed for several purposes such as for dosage adjustment in ADHD patients, clean validation in the pharmaceutical industry, and for trace determination in forensics.

***Keywords*:** Microextraction, Atomoxetine, Plasma and urine samples, Minitab, ADHD, ANN.

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Introduction

Atomoxetine (Strattera), (-)-N-Methyl-3-phenyl-3-(o-tolyloxy)-propylamine hydrochloride, is a selective norepinephrine reuptake inhibitor, used clinically for the treatment of attention-deficit hyperactivity disorder (ADHD) in children, adolescents, and adults. However, its efficacy has not been studied in children under six years old. Its advantage over stimulants for the treatment of ADHD is that it has less abuse potential and is not scheduled as a controlled substance. Since norepinephrine is believed to play a role in ADHD, atomoxetine was tested – and subsequently approved – as an ADHD treatment agent [1]. Atomoxetine may be quantitated in plasma, serum or whole blood in order to distinguish extensively versus poor metabolizers in those receiving the drug therapeutically, in order to confirm that the potential poisoning victims can be diagnosed or assist in the forensic investigation in case of fatal overdosage[2]; therefore, developing reliable analytical methods for routine monitoring of atomoxetine, which are rapid, precise, and economical in cost and time, is essential.

Several liquid chromatography methods with ultraviolet and mass spectrometer detection have been reported for the analysis of atomoxetine in biological fluids. However, some analytical techniques do not have an adequate limit of detection (LOD) suitable for monitoring atomoxetine at low concentrations [3]. LC/MS/MS methods have shown improvement in sensitivity, however, the mass spectrometer is very expensive [4-7]. Therefore, due to the low concentration of atomoxetine in plasma samples, ranging from 0.6 to 1065.7 ng ml^{-1} after administration of different dosage regimens [8], pretreatment and the pre-concentration step is generally required before determination of trace amounts of the drug. Several extraction and preconcentration techniques such as liquid-liquid extraction (LLE) [9], solid phase extraction (SPE) [11, 12], liquid phase microextraction (LPME) [12,13] and solid phase microextraction (SPME) [14-16] were used for sample preparation. LLE has main drawbacks such as being time-consuming, generally, labor-intensive and necessitates large quantities of toxic, expensive, and environmentally unfriendly organic solvents. The solvent microextraction effectively overcomes these problems by reducing the amount of organic solvent. To improve the stability and reliability of liquid–phase microextraction (LPME), Pedersen-Bjergaard and Rasmussen introduced hollow fiber liquid phase microextraction (HF-LPME)[17]. HF-LPME is one of the effective solvent microextraction techniques that have been discussed in several papers [16, 17, 18]. HF-LPME employed hollow fiber to contain and protect the extraction solvent during extraction. Therefore, it is much more robust than single drop microextraction (SDME) because the micro drop suspended on the needle of microsyringe may be dislodged during the extraction, especially when samples are stirred vigorously [17,19]. It is an effective and reliable technique that provides high preconcentration factor, short extraction time and excellent sample

clean-up capability. It has been applied for the analysis of several organic and inorganic species in different biological and environmental samples with complex matrices [25-33].

In the present study, a simple and efficient SBME method in combination with HPLC-UV was developed for preconcentration and determination of atomoxetine in biological fluids [24]. To our knowledge, this is the first report of the use of SBME combined with HPLC-UV for the trace analysis of atomoxetine in human urine and plasma.

Experimental

Chemicals and materials

Atomoxetine reference standard was kindly donated by Food and Drug Organization (Tehran, Iran). HPLC-grade methanol, acetonitrile, potassium dihydrogen orthophosphate, and orthophosphoric acid were purchased from Merck (Darmstadt, Germany). n-octanol, n-heptanol, n-decanol, isobutyl methyl ketone (IBMK), HCl, KCl, and NaOH were bought from Merck (Darmstadt, Germany). All the other chemicals were of reagent grade or of the highest purity available. Phosphate buffer was prepared from phosphoric acid and their appropriate salts (Merck Chemical Co.). Ultrapure water (resistivity, 18.2 MΩ cm^{-1}) was produced by a Milli-Q system (Millipore, USA), and was used throughout for the preparation of solutions. Frozen, drug-free human plasma was obtained from Iranian Blood Transfusion Organization (Tehran, Iran) and the urine samples were obtained from the Clinic of Taleghani Hospital (Tehran, Iran). The polypropylene hollow fiber (200μm wall thickness, 600μm internal diameter, 0.2 μm pore size) was purchased from Membrana (Wuppertal, Germany). The stock standard solution of atomoxetine (100 mg L^{-1}) was prepared in methanol and stored in the refrigerator (4 ◦C). All of the working standard solutions were freshly prepared by proper dilution of the stock standard solution with ultrapure water to the required concentration.

Apparatus and chromatographic conditions

Chromatographic analysis was performed by Shimadzu (Japan) HPLC system equipped with an LC_10ADVP quaternary pump and a UV/VIS SCL_10 AVP detector. Chromatographic data were recorded and analyzed using Class VP software. The chromatographic separation was carried out at room temperature (about 25 ◦C) on a C18 analytical column (150 mm × 4.6mm, 5μm) with a C18 guard column (4.0 mm × 10mm, 5 μm) from Teknokroma (Barcelona, Spain), an isocratic pump, a 10μl injection loop and a PDA detector. Phosphate buffer: acetonitrile (70:30, v/v) was used as mobile phase at a flow rate of 1 ml/min. Phosphate buffer was prepared freshly by dissolving 1.36g of potassium dihydrogen orthophosphate in 1000 mL water and the the pH of the solution was adjusted to 2.5 (±0.05) with a dilute orthophosphoric acid solution. The solution was filtered

through a 0.22μ cellulose acetate filter. The analyte was detected at 224 nm. All of the pH measurements were performed with a GPHR 1400A pH meter (Germany). Stirring of the solution was carried out with a Heidolph MR 3001 K magnetic stirrer (Schwabach, Germany) and a (7 mm × 1.5 mm) magnetic stirring bar. A 50 μL syringe model 702 NR from Hamilton (Bonaduz, Switzerland) was used.

SBME procedure

A 100 mg L^{-1} stock solution of atomoxetine was prepared in water and standard working solutions were prepared by the spiking proper amount of the stock solution in pure water. Hollow fiber was cut into 4.5 cm pieces and then washed in acetone in the ultrasonic device and was dried at room temperature. Using Hamilton syringe, Acceptor phase with a pH of 2.5 was injected into the hollow fiber, which was immersed in n-octanol. After removing the excess amount of the acceptor phase, both ends of the hollow fiber was sealed mechanically using small aluminum foil pieces. Then it was placed in a beaker containing 10 ml donor solution containing imatinib. The beaker was placed on a stirrer after putting a magnetic stirrer bar in the beaker. The temperature was 25°C. After 25 min, the solvent bar was unsealed and the acceptor solution was drawn into a Hamilton syringe for an injection to HPLC-UV for detection [10, 20-24].

Statistical analysis

The data obtained from the experiments were analyzed using Minitab version 15.11. Software. Response surface methodology [33] was chosen for the analysis of data obtained from the experiments. Six parameters were optimized, accordingly. The maximum (+1), center (0), and minimum (-1) ranges were studied which can be seen in Figure 1. The 3D figures show the interaction of each parameter on each other, 2 by 2, while the other factors were held (Figure 1).

Real Samples

Urine and plasma samples were taken from the volunteer patients.

Calculation of PF, Relative recovery, and extraction recovery

The ratio of the final analyte concentration in the acceptor phase ($C_{f,a}$) and the initial concentration of the analyte ($C_{i,s}$) in the sample solution is defined as a preconcentration factor:

$$PF = \frac{Cf,a}{Ci,s} \quad (1)$$

where $C_{f,a}$ was calculated from a calibration graph obtained by direct injection of analytes standard solutions. Extraction recovery (*ER*) was defined as the percentage of the number of moles of analyte which was extracted to the acceptor phase ($n_{f,a}$) divided by the number of moles of analyte originally presented in the sample solution ($n_{i,s}$).

$$ER = \frac{n_{f,a}}{n_{i,s}} \times 100 = \frac{n_{f,a} \times V_{f,a}}{n_{i,s} \times V_{i,s}} \times 100 \qquad (2)$$

$$ER = (\frac{V_{f,a}}{V_{i,s}}) PF \times 100 \qquad (3)$$

Where $V_{f,a}$ and $V_{i,s}$ are the volumes of acceptor phase and sample solution, respectively.

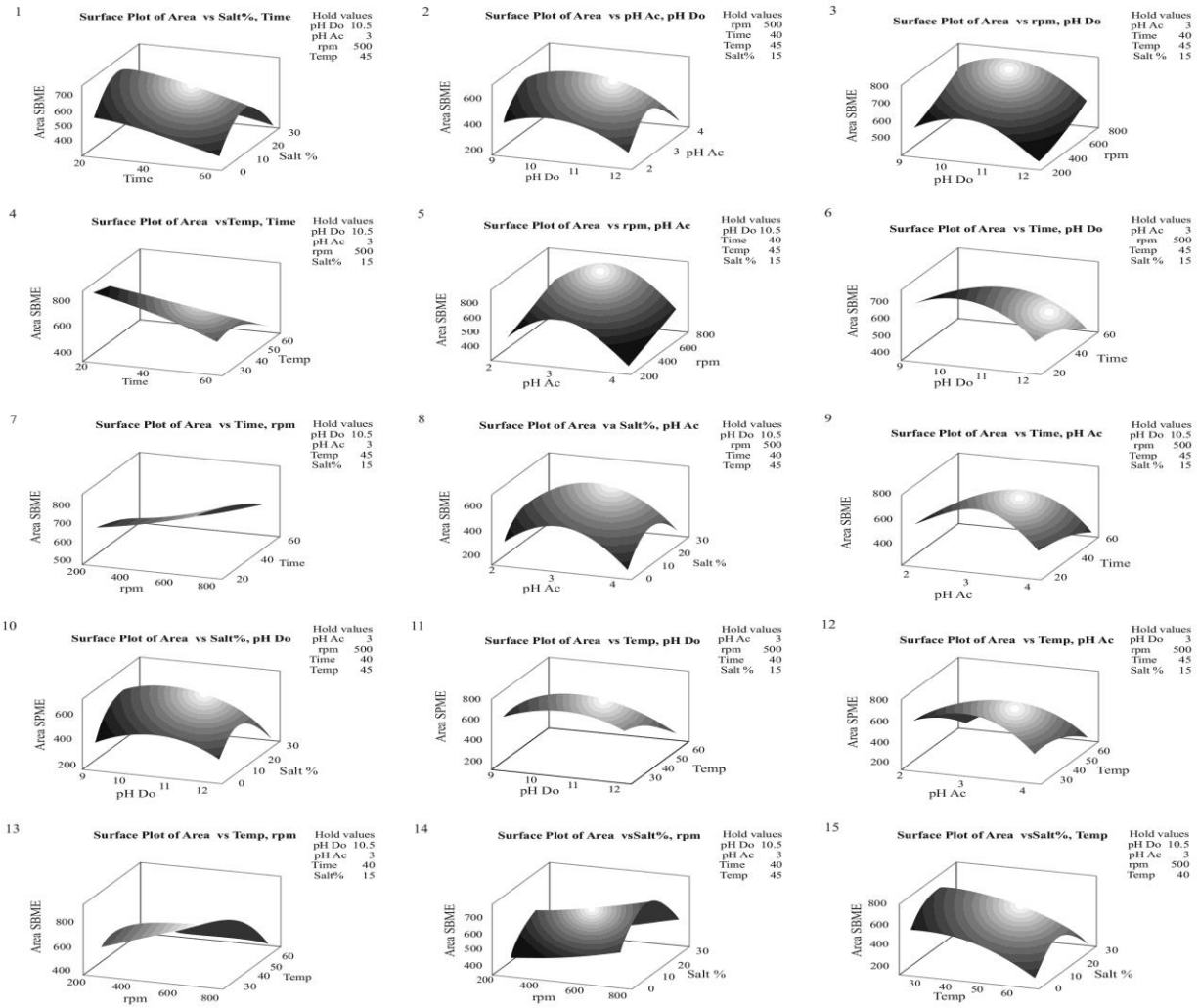

Figure 1. Response surface methodology showing the interaction of different parameters on the optimum microextraction of Atomoxetine.

Results and discussion

In this experiment, a three-phase hollow fiber microextraction method combined with HPLC-UV was applied for the extraction and determination of atomoxetine in plasma and urine samples. In this method, some important extraction parameters such as the type of organic solvent, pH of donor phase and acceptor phase, ionic strength, stirring rate, extraction time and temperature were studied and optimized.

Selection of organic extraction solvent

The type of organic solvent to be immobilized within the pores of the HF is important to ensure satisfactory analyte preconcentration. The chosen organic solvent must be easily immobilized within the pores of the fiber, have a high selectivity for the analyte and low tendency to extract the interferences existing in the donor phase, immiscible with water to avoid dissolution and be nonvolatile to prevent solvent loss during the extraction. In this study, the effect of different organic solvents such as n-octanol, n-decanol, n-heptane, and isobutyl methyl ketone (IBMK) was investigated on the extraction efficiency of atomoxetine. n-octanol can provide the best extraction performance for its much higher enrichment factor and selectivity and low solvent loss. Therefore, n-octanol was selected for the rest of the work.

The pH of donor and acceptor phase

The pH of both the donor phase and the acceptor phase affect the extraction performance. For basic drugs, the donor phase should be strongly alkalized to effectively demonize the analytes and consequently reduce their solubility within the sample; while, the acceptor phase should be acidized in order to promote dissolution of the basic analytes [20]. For practical applications, pH should differ from the pK_a value of the analytes (pK_a of atomoxetine is 9.8) by at least 2 or 3 units. In this study, the effect of pH of the donor phase was investigated ranging from 9 to 12. It was found that the extraction efficiency increased when the pH was fixed at 10.3. Thus, a pH of 10.3 for the donor phase was chosen for subsequent experiments. Also, the effect of the pH of the acceptor phase on the extraction efficiency of atomoxetine was investigated in the range of 2-4. Finally, the pH of 3.0 was selected as the optimum pH value.

Effect of stirring rate

Agitation of the sample is routinely applied to accelerate the extraction kinetics. Facilitating the diffusion rate of the drug from the aqueous sample to the organic phase is achieved by increasing

the stirring speed of the aqueous phase. In this work, the effect of stirring rate on the extraction of target drug was investigated by agitating 10 mL sample solution at different stirring rates (200-800 rpm) using magnetic stirrer. The obtained results show that the extraction efficiency of the drug increased by increasing the stirring rate up to 356 rpm.

Salt effect

In this work, the effect of salt addition on the extraction efficiency of atomoxetine using HF-LPME method was examined by adding KCl to the aqueous samples in the range of 0–30% (w/v). The salt concentration of 15.1% (w/v) was selected as the optimum concentration for subsequent experiments. The salting-out effect (this can increase the efficiency of the extraction) and electrostatic interaction between salt and analyte could occur when adding salt. In addition, adding salt can increase the viscosity of the sample solution and change the physical properties of the fiber wall. These interactions can reduce the movement of analyte from the donor phase to the solvent. Generally, adding salt may increase, decrease or not change the efficiency depending on the nature of analyte [21].

Effect of extraction time and temperature

The effect of extraction time in the range of 20-60 min was evaluated. The experimental results demonstrated that the extraction efficiency increased when increasing the extraction time at 25 min, but decreased thereafter. Generally, if extraction time is too long, solvent loss and air bubble formation on the surface of the hollow fiber may occur when both of them decrease the amount of analyte extracted. Consequently, 26 min was selected as the optimal extraction time.

The temperature has a significant effect on both the kinetics and the thermodynamics of the extraction process. To study the effect of extraction temperature on HF-LPME, the extraction responses were investigated over a temperature range of 20-60 °C. Increasing temperature can promote the mass transfer coefficient which improves the extraction efficiency in a shorter time. On the other hand, the solvent loss may occur due to increased solubility of the organic solvent at higher temperatures, which declines the extraction efficiency. The obtained results indicate that the peak area ratio of the analyte reaches its maximum at about 25 °C.

Analytical Performance

Under optimized conditions, the analytical performance of the developed method such as LOD, LOQ, and RSD% was evaluated and summarized in Table 1. The calibration curve was obtained

after the standard series were subjected to the HF-LPME-HPLC. A broad dynamic linear range with good correlation coefficient (r) was achieved. Limit of detection (LOD) and limit of quantification (LOQ) was calculated as the minimum concentration of an analyte giving peaks whose signal-to-noise ratio is 3 and 10, respectively.

Table 1. The experiments designed for SBME and HFLPME of atomoxetine and the areas obtained using HPLC-UV.

No	The pH of the donor phase	The pH of acceptor phase	Stirring rate (rpm)	Time (min)	Temperature (°C)	Salt W/V%	Area for SBME	Area for HFLPME
1	9	2	250	20	25	0	248	260
2	9	2	250	20	45	15	318	335
3	9	2	250	20	65	30	109	134
4	9	3	500	40	25	0	331	302
5	9	3	500	40	45	15	835	927
6	9	3	500	40	65	30	81	139
7	9	4	750	60	25	0	96	82
8	9	4	750	60	45	15	212	269
9	9	4	750	60	65	30	95	111
10	10.5	2	500	60	25	15	362	509
11	10.5	2	500	60	45	30	128	139
12	10.5	2	500	60	65	0	57	80
13	10.5	3	750	20	25	15	1150	1355
14	10.5	3	750	20	45	30	560	496
15	10.5	3	750	20	65	0	197	151
16	10.5	4	250	40	25	15	248	290
17	10.5	4	250	40	45	30	156	175
18	10.5	4	250	**40**	65	0	47	39
19	12	2	750	40	25	30	317	364
20	12	2	750	40	45	0	218	296
21	12	2	750	40	65	15	31	43
22	12	3	250	60	25	30	133	168
23	12	3	250	60	45	0	260	241
24	12	3	250	60	65	15	30	34
25	12	4	500	20	25	30	65	86
26	12	4	500	20	45	0	140	103
27	12	4	500	20	65	15	53	72

Table 2. Figures of merit of SBME and HFLPME of atomoxetine.

	LOD (ng mL^{1})	LOQ (ng mL^{-1})	Linearity (ng mL^{-1})	R^2	PF[a]	RSD% [b]	
						Within-day	Between day
SBME	7.0	20.0	20.0-6000.0	0.99	107	4.9	5.7
HFLPME	7.0	20.0	20.0-6000.0	0.99	92	4.9	6.4

Analysis of plasma and urine samples

Under the optimized conditions, the developed SBME-HPLC technique was applied for preconcentration and determination of atomoxetine in plasma and urine samples (Figure 2).

A comparison between the analytical characteristics of the developed method and those of the published methods was shown in Table 3. As can be seen, the LOD of this method is comparable with those obtained in the previous studies and even lower than those reported in the literature [20]. Although the developed method has less sensitivity than those reported in references [18, 19, 20], those methods are more expensive and the instrumentation is not available in most laboratories. In addition, due to the simplicity and low cost of the extraction device, the hollow fiber can be discarded after each extraction to avoid carryover and cross-contamination. Finally, it is concluded that this method is an effective technique for the preconcentration and determination of atomoxetine in plasma and urine samples.

Table 3. The relative standard deviation obtained by SBME-HPLC-UV for atomoxetine.

Sample	***Cadded*** **(mg L^{-1})**	***Cfound*** **(mg L^{-1})**	**RSD% (n = 5)**	**RR%**
SBME-Plasma	1	0.84	5.3	84
SBME-Urine	1	0.91	4.4	91
HFLPMEPlasma	1	0.78	6.5	78
HFLPME-Urine	1	0.83	5.8	83

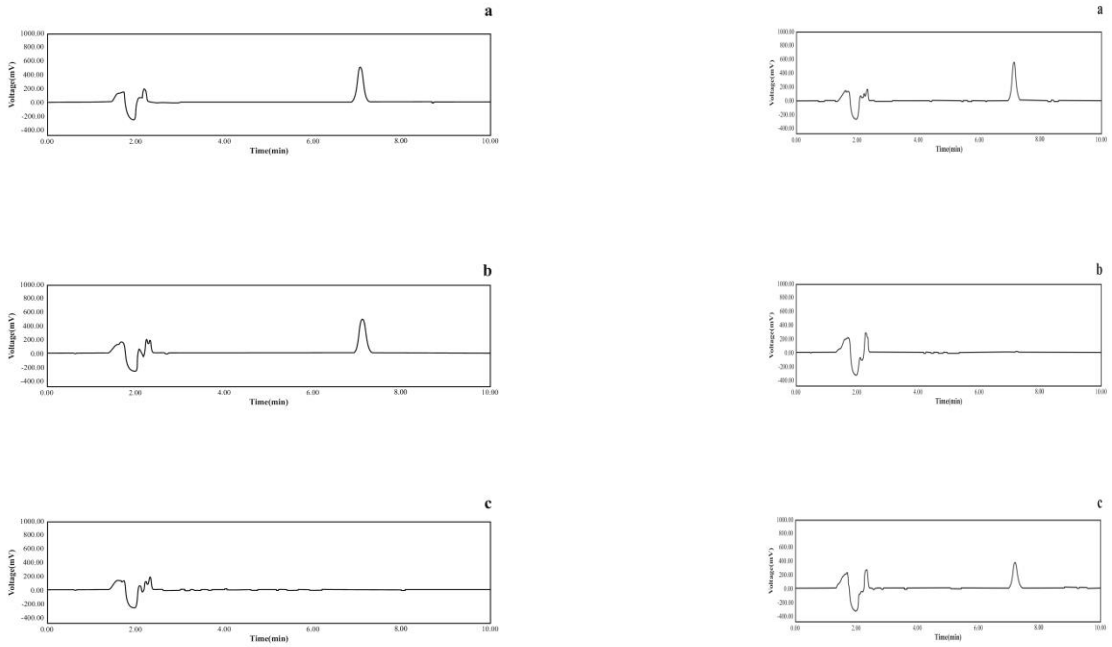

Figure 2. The chromatograms of (left) plasma sample before SBME (c) plasma sample containing atomoxetine after SBME (a and b), urine sample (right): urine sample before SBME (b) and after SBME under optimal conditions (a and c).

Modeling the data using artificial neural networks

In the end, laboratory data were predicted using artificial neural networks with 10 neurons and a correlation coefficient of 0.9643 and a total squared error of 1.888, which resulted in a successful prediction.

The input parameters defined for the artificial neural network are the condition of the experiment such as pH of donor and acceptor phase, stirring rate, salt addition, temperature and time and the target or output function is the same area obtained by HPLC-UV that is used. To develop and validate this extended model, the entire database was randomly divided into three sections: 70%, 15%, and 15% of the data points were used as training, validation, and test respectively. In order to improve the performance of the artificial neural network, input values and targets were normalized in the range from -1 to 1. The number of neurons in each secret layer was different, and each network was repeatedly executed for 10 times. The accuracy of the estimation model based on the artificial neural network was defined by the correlation criterion between predicted and real values. As shown in Figure 3, this factor is used for all three parts of the training, validation, and test, and

its value is calculated by the network. The values of the correlation coefficient for the total data are also obtained.

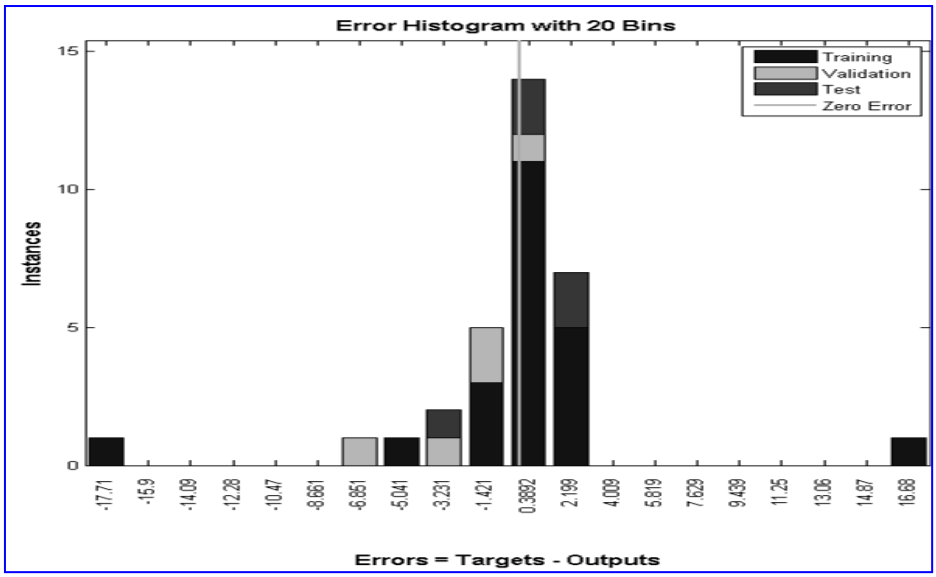

Figure 3. Error values (differences in the laboratory and predicted values) for training, validation, and test data.

The values are equal to 0.92175, 0.99421, 0.98673 for three parts of the training, validation, and test, which indicates the successful prediction of the network with 10 neurons. However, this amount for the whole data is equal to 0.94384, which indicates a successful prediction.

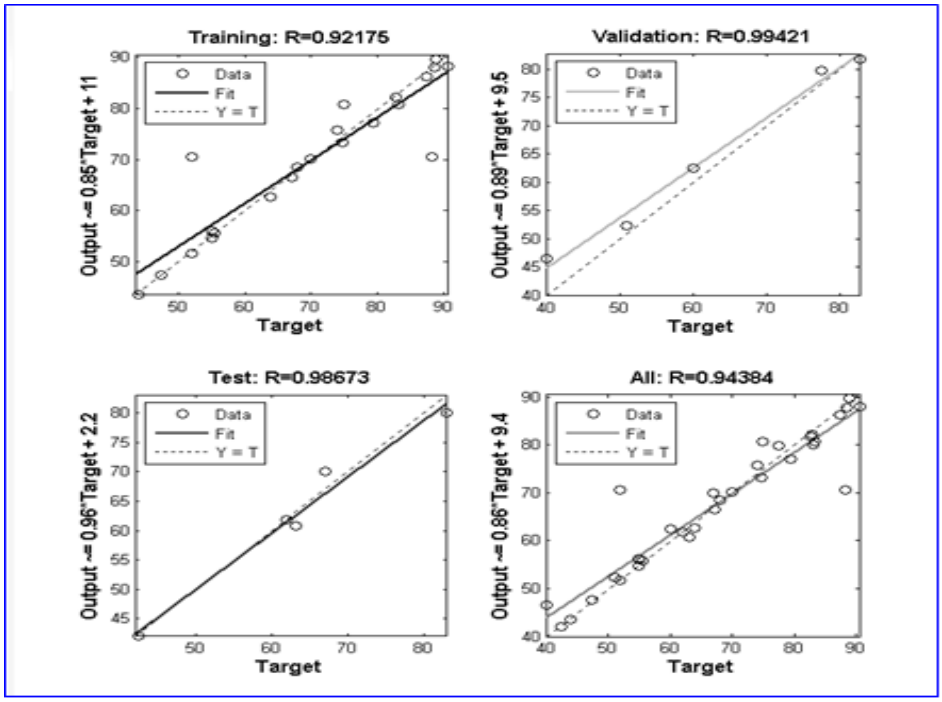

Figure 4. Correlation coefficients for the implemented neural network.

As shown in Figure 4, there is a histogram for the efficiency in which the x-axis is the difference between the experimental and predicted values for the network. As you can see, all data is near the Zero Error line, and the error distribution is very low. The maximum and minimum error values are -17 and 16. Finally, laboratory data were predicted using artificial neural networks with 10 neurons and a correlation coefficient greater than 0.96 and a square error magnitude of 1.8. The results of this prediction were very successful.

Conclusions

It can be concluded from the results, that SBME is a selective, cost-effective, and practical method for preconcentration of atomoxetine prior to HPLC-UV detection. Also, this approach will help monitor the dose of the drug in blood and urine samples for critical patients. Medical centers can easily benefit from such sample treatment method for accurate results.

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