Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Simultaneous Determination of Amphetamine, Ephedrine, and Ascorbic Acid in Human Serum Using a Validated RP-HPLC-PDA with Artificial Neural Network Modeling: Application in Clinical Toxicology

Fatima Abdul Rasool

as.24.43@grad.uotechnology.edu.iq

University of Technology

Rulla Sabah

Mustansiriyah University

Ahmed Jassim Muklive

University of Technology

Research Article

Keywords: amphetamine, ephedrine, ascorbic acid, method validation, false-positive, forensic toxicology, Artificial Neural Network

Posted Date: October 28th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11253842/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Simultaneous Determination of Amphetamine, Ephedrine, and Ascorbic Acid in Human Serum Using a Validated RP-HPLC-PDA with Artificial Neural Network Modeling: Application in Clinical Toxicology

Fatima Abdul Rasool[1*]. Rulla Sabah[2]. Ahmed Jassim Muklive [1]

[1]Department of Chemistry, Applied Science, University of Technology, Baghdad 10066, Iraq.

[2]Department of Chemistry, College of Science, Mustansiriyah University, Baghdad, 10011, Iraq.

[*]Corresponding author: Fatima Abdul Rasool, as.24.43@grad.uotechnology.edu.iq

Abstract

A rapid, highly sensitive method using reversed-phase high-performance liquid chromatography with photodiode array detection (RP-HPLC-PDA) was developed to simultaneously quantify and qualify amphetamine (AMP), ephedrine (EPH), and ascorbic acid (vitamin C) in human serum. Chromatographic separation conditions was obtained on a pH stable hybrid silica C18 column (250 mm × 4.6 mm, 5 µm) which is stable up to pH 12 using a time programmed gradient mode for mobile phase system acetonitrile: methanol combined with orthophosphoric acid (pH 9.0, amphetamine phase), ammonium formate/formic acid (pH 9.0, ephedrine phase), and a tetrabutylammonium-modified phosphate buffer at pH 4.0 for ascorbic acid, within a total analytical run of less than 14 minutes. A multilayer feed-forward artificial neural network (ANN) with a 3-10-3 topology was developed in MATLAB to model the relationship between the chromatographic parameters (mobile phase ratio, pH, and flow rate) and the responses (retention time, peak area, and tailing factor). The method exhibited good linearity (R^2 = 0.9999) over 5.0-25.0 ng. mL^{-1} for all three substances. Limits of detection (LOD) were 0.195, 0.164, and 0.116 ng. mL^{-1}, and limits of quantification (LOQ) were 0.593, 0.497, and 0.352 ng. mL^{-1} for AMP, EPH, and ascorbic acid, respectively. Precision and accuracy studies showed %RSD values below 0.90% and recovery percentages ranging from 99.7% to 100.3%, indicating the stability of the pH-adjusted mobile phases. The ANN model showed strong agreement between experimental and predicted values, with correlation coefficients (R) of 0.925, 0.975, and 0.987 for the training, validation, and test sets, respectively**.** Application of the validated method to human serum samples revealed a statistically significant decrease in serum ascorbic acid levels among addiction compared to healthy controls (3526 ± 110.94 ng. mL^{-1} vs. 8718 ± 354.04 ng· mL^{-1}, $p < 0.0001$). Monitoring serum levels during a 60-day detox program explains the near-complete release of AMP and EPH alongside substantial recovery of ascorbic acid levels. This RP-HPLC-PDA method was sensitive, precise, and practical for clinical toxicology and therapeutic monitoring**.**

Keywords: amphetamine; ephedrine; ascorbic acid; method validation; false-positive; forensic toxicology; Artificial Neural Network.

1. Introduction

The analytical detection of amphetamine, a broad class of synthetic phenethylamine derivatives that act as potent central nervous system (CNS) stimulants by modulating or changing neurotransmission function, achieving ultimate diagnostic specificity remains as a challenge in clinical toxicology and forensic crime scene [1,2]. Amphetamines are characterized by the α-methylphenethylamine structure and are indicated for the treatment of attention deficit and hyperactivity disorder (ADHD) and narcolepsy. The preliminary detection via immunoassay-based urine drug screens, which is a rapid competitive binding test that employs antibody-antigen kinetics, is compromised by high false-positive rates reported as 3.9% and 9.9% in the literature [3]. These false-positive analytical assays have illustrated socioeconomic and judicial consequences, ranging from the termination of employment to protracted legal disputes and the systemic impact on addicted patients [4]. While immunoassays are still the primary screening modality because of their high assay capacity, their dependence on the paratope–epitope binding affinity of specific antibodies makes them to form a susceptible cross-reactivity with exogenous structural analogs [1,5]. Ephedrine, considered a sympathomimetic alkaloid and β-hydroxy-phenethylamine used as a decongestant, stills as a principal of this interference to its substantial structural with the phenethylamine drugs [6,7]. The challenge of structural similarity and chemical interference from vitamin C (L-ascorbic acid), a six-carbon lactone and essential water-soluble antioxidant, is a further threat to the integrity of enzymatic assays. As a potent biological reducing agent, L-ascorbic acid illustrates the reduction of chromogenic substrates, thereby disrupting the redox signaling in many colorimetric and peroxidase-linked immunoassays [8], [9]. Elevated concentrations of urinary ascorbic acid have further been implicated in diagnostic error through instability of assay kinetics or pH-effect shifts in antibody-antigen binding thermodynamics [10]. To mitigate these analytical risks, orthogonal confirmatory testing is an essential requirement for forensic defensibility [1]. Chromatographic techniques, including thin-layer chromatography (TLC), gas chromatography–mass spectrometry (GC-MS), and liquid chromatography tandem mass spectrometry (LC-MS/MS), constitute the principal confirmatory approaches in this domain, yet each presents distinct practical limitations. TLC, despite its operational simplicity, lacks the sensitivity and quantitative precision required for rigorous toxicological analysis. Although GC-MS has historically served as the definitive confirmatory standard, its reliance on time-intensive pre-column derivatization typically acylation or sialylation to reduce the polarity of primary amines constitutes a significant operational bottleneck [11,12]. LC-MS/MS, while offering high sensitivity and specificity, remains costly and requires specialized expertise that limits its accessibility for routine screening in many clinical and forensic laboratories[13,14]. High-performance liquid chromatography (HPLC), by contrast, enables the direct analysis of polar stimulants without derivatization, offering a practical and cost-effective balance of sensitivity, resolving power, and operational simplicity required to discriminate structurally related compounds on the basis of their physicochemical properties [15,16,17].

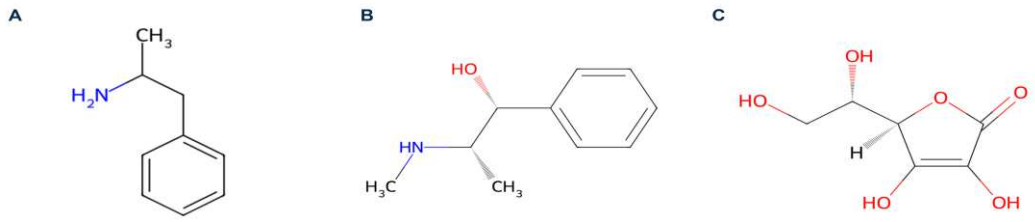


Fig 1. chemical structure of **A:** Amphetamine (Amp), **B:** Ephedrine (Eph), **C:** Ascorbic Acid (Vit.C).

2 Materials and Methods

2.1 Chemicals and Reagents

Amphetamine and ephedrine reference standards were obtained from the National Center for Drug Control and Research (Baghdad, Iraq). Ascorbic acid (vitamin C) reference standard was purchased from Sigma-Aldrich (Darmstadt, Germany). Acetonitrile and methanol were of HPLC grade (≥99.9%). Orthophosphoric acid, formic acid, ammonium formate, and tetrabutylammonium hydrogen sulfate were of analytical grade (≥99%). High-purity water was obtained using a Milli-Q water purification system (Millipore, USA) and used throughout the study. All chemicals and reagents were used as received without further purification.

2.2 Apparatus

Chromatographic separation and quantitative evaluation were performed using an HPLC system (Sykam GmbH, Germany). The instrumentation comprised a high-pressure triplex gradient solvent delivery pump, an inline vacuum degassing unit for mobile phase deaeration, and a high-sensitivity photodiode array (PDA) detector. High-pressure solvent mixing was accomplished via a T-shaped coupling interface coupled with a stainless-steel capillary mixing loop to ensure complete mobile phase homogenization prior to column delivery. Analytical separation was achieved on a C18 stationary phase column (250 mm × 4.6 mm, 5 µm particle size). System control, data acquisition, and peak integration were executed using Sykam chromatographic software.

2.3 Preparation of Standard and Calibration Solutions

Stock solutions of 400 µg. mL^{-1} of amphetamine, ephedrine, and vitamin C were prepared by dissolving 0.1000 g of each standard drug in deionized water, then bringing the volume to 250 mL in a volumetric flask. After that, a series of working calibration standards, ranging from 5 to 25 ng/mL for each analyte by serial dilution. All solutions were degassed by ultrasonication for 15 to 20 min and filtered through a 0.45 µm syringe filter prior to HPLC injection.

2.4. Chromatographic Conditions

2.4.1. Selection of Optimal Detection Wavelength

To determine the optimal wavelengths for individual standard solutions of amphetamine, ephedrine, and vitamin C, the solutions were scanned over the UV spectral range (200–400 nm) using a UV-Vis spectrophotometer. Depending on the resulting absorption maxima (λmax), wavelengths of 260 nm, 270 nm, and 280 nm were selected for amphetamine, ephedrine, and vitamin C, respectively. Multi-wavelength parameters were programmed into the HPLC-PDA system for the simultaneous qualitative and quantitative analysis of each substance at its maximum absorbance within a single chromatographic run time.

2.4.2. Chromatographic Conditions

Chromatographic separation and simultaneous quantification of amphetamine, ephedrine, and vitamin C within a single analytical injection as a mixture were performed using a gradient elution mode on a C18 column (25 cm × 4.6 mm, 5 μm). The mobile phase flow rate was 0.7 mL. min^{-1} all over the chromatographic run. The gradient mode and multi-wavelength detection using the photodiode array (PDA) detector were operated as follows:

Preparation of Mobile Phase:

Amphetamine Elution Phase (0.01–5.00): Prepared by mixing an organic phase of acetonitrile and methanol (80:20, v/v) with an aqueous fraction whose pH was adjusted to 9.0 using dilute orthophosphoric acid and sodium hydroxide. The stable signal was maintained at 260 nm.

Ephedrine Elution Phase (5.01 – 7.00): Prepared using acetonitrile and water (90:10, v/v) containing 5 mM ammonium formate and 0.1% (v/v) formic acid. The buffer pH was adjusted to 9.0 using dilute sodium hydroxide. The stable signal was maintained at 270 nm.

Vitamin C Elution Phase (7.01 – 14.00): mixture of methanol and an aqueous pH phosphate buffer (80:20, v/v), with tetrabutylammonium (TBA) as an ion-pairing reagent to support retention of the strongly polar ascorbic acid on the non-polar C18 stationary phase. The aqueous phase pH was adjusted to 4.0 with dilute orthophosphoric acid. Stable signal detection was at 280 nm.

2.5. Method Optimization

An optimization of the experimental parameters was performed to obtain optimal analysis efficiency, resolution, and peak symmetry. The influence of variables such as mobile phase ratio, pH, and flow rate on separation efficiency was determined using a mixture of standard solutions at 5 ng. mL^{-1} for each analyte under simultaneous gradient elution conditions.

2.5.1. Effect of Mobile Phase Ratio

The optimal eluent chromatographic selectivity was studied by systematically changing the mobile phase ratio (organic to aqueous) (50:50, 60:40, 70:30, 80:20, and 90:10, v/v) at an optimum pH of 7.0 and flow rate. Optimal resolution and peak symmetry were obtained at ratios of 80:20 (v/v) for Amphetamine, 90:10 (v/v) for Ephedrine, and 80:20 (v/v) for Vitamin C.

2.5.2. Effect of pH

After optimization of the mobile phase ratio, the effect of pH on substance ionization, retention time, and peak tailing was studied at different pH values of 4.0, 7.0, and 9.0. Optimal resolution, separation efficiency, and peak symmetry were obtained at pH 9.0 for both amphetamine and ephedrine, and at pH 4.0 for ascorbic acid.

2.5.3. Effect of Flow Rate

The effectiveness of mobile phase flow rate on resolution, column backpressure, peak symmetry, and sharpness was investigated by range of 0.5, 0.7, 1.0, 1.2, and 1.5 mL min^{-1}. A flow rate of 0.7 mL min-1 was selected as the optimum mobile flow rate, indicating the best balance between peak sharpness, retention time, and run time for the analysis

.2.6. Method Validation Procedure

The analytical performance of the HPLC-PDA method was validated with the ICH Q2(R2) Guidelines (2024), ensuring that the requirements for forensic and clinical application [18]. The validation included the evaluation of linearity, sensitivity, accuracy, and precision across the target analytical substance [19]

2.6.1. Linearity, LOD, and LOQ

Linearity was established over a concentration range of 5–25 ng. mL^{-1}. Method sensitivity was quantified through evaluation of the limit of detection (LOD) and limit of quantification (LOQ), which were obtained statistically from the standard deviation of the response (σ) and the slope of the calibration curve (S), using the formulas LOD = 3.3σ/S and LOQ = 10σ/S [18,20]. This ensures that the method remains robust against baseline noise while indicating a high-resolution quantification at trace analyte levels [21].

2.6.2. Accuracy and Precision

Accuracy was evaluated via recovery studies using standard substance concentrations at 7.0, 12.0, 17.0, and 23.0 ng. mL^{-1} within the linear range, with triplicate determinations. Precision was assessed as repeatability (intra-day, n = 3) and intermediate precision (inter-day, over three consecutive days, n = 3), evaluated as relative standard deviation (%RSD) [22].

2.6.3 Artificial Neural Network Modeling and Optimization

To optimize the chromatographic parameters, an Artificial Neural Network (ANN) process was applied using MATLAB software. A multi-layer feed-forward neural network with a 3-10-3 topology was designed. The input layer consisted of three operational parameters such as Mobile Phase Ratio, pH, and Flow Rate, while the output layer contained three neurons of the analytical responses such as Retention Time, Peak Area, and Tailing Factor. A hidden layer consisting of ten processing neurons supplied with weight matrices, bias vectors, and non-linear transfer functions was employed to capture complex interactions. The experimental dataset was imported into MATLAB, and the network was trained using standard fitting algorithms. The model's predictive performance, accuracy, and generalization capability were thoroughly assessed based on the Mean Squared Error (MSE) and linear regression correlation coefficients, such as R-values across training, validation, and test datasets.

2.7. Clinical Study Design and Participant Selection

This prospective, longitudinal clinical and analytical study was designed to quantify drug concentration dynamics and evaluate chromatographic separation of target analytes using serum HPLC, applying the method developed and validated in Sections 2.1-2.6. A total cohort of 100 male participants (N = 100) was enrolled and categorized into four frequency-matched groups
(n = 25 per group):

- **Group I (Healthy Control):** Age- and lifestyle-matched, drug-free volunteers with no clinical evidence of substance use and negative drug screens.
- **Group II (Amphetamine-Addicted):** Diagnosed amphetamine-dependent patients admitted to an addiction treatment and rehabilitation center.
- **Group III (Ephedrine-Addicted):** Gym members and athletes reporting illicit or off-label ephedrine use as a stimulant/performance enhancer, with no history of amphetamine use, subsequently diagnosed with ephedrine dependence and admitted for treatment.
- **Group IV (Amphetamine + Ephedrine Co-Addicted):** Polydrug users admitted with co-ingestion patterns showing concurrent amphetamine and ephedrine dependence.

Patients in the addicted groups (Groups II–IV) were diagnosed by experienced psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) criteria. For longitudinal evaluation, baseline serum samples were systematically collected during the initial admission window (within Days 1–3 of hospitalization), followed by a second post-recovery evaluation after 60 days of standardized detoxification. Healthy control participants (Group I) were evaluated at a single baseline time point to establish healthy reference serum matrices.

2.8. Inclusion and Exclusion Criteria

Patients undergoing their first attempt at addiction rehabilitation therapy under a standardized clinical regimen were eligible for inclusion. Patients admitted to the addicted groups **(**Groups II–IV) routinely received antioxidant supplementation, including vitamin C, as part of the standardized in-hospital clinical treatment protocol from the time of admission; given the central role of vitamin C as the primary biochemical marker of interest in this investigation, vitamin C was specifically selected for quantitative monitoring among the antioxidants administered [23,24]. To minimize metabolic confounding, individuals with pre-existing severe hepatic, renal, cardiovascular, hematological, or autoimmune disease were excluded, as were participants receiving therapeutic agents known to affect core metabolic or biochemical pathways other than the study substances [25,26].

2.9. Serum Sample Collection, Processing, and Storage

A total of 175 serum samples were collected: 100 baseline samples and 75 post-recovery samples. About 5 mL of whole blood was collected into sterile tubes. Re-collected from the addicted groups II–IV, n = 75, 25 per group, following the 60-day recovery phase. Blood specimens were allowed to clot at room temperature for 30 min, then centrifuged at 1500 rpm for 5 min [27]. The sera were immediately collected into sterile microcentrifuge tubes and preserved at −25 °C until HPLC analysis [28]. Before HPLC analysis, the treated serum extracts were appropriately

diluted using mobile phase A as a diluent to bring the concentrations of the analyte within the established linear range (5.0-25.0 ng/mL). Specifically, the drug samples were diluted 1:20, and the basic ascorbic acid samples were diluted 1:400. The use of mobile phase A as a diluent ensured optimal peak congruence and prevented solvent peak distortion during gradient initiation. All clinical serum concentrations listed in Table 6 represent final values corrected for the dilution factors

2.10. Serum Analysis by HPLC

To quantify the presence of amphetamine, ephedrine, and vitamin C, serum samples were thawed at room temperature and analyzed using the validated HPLC-PDA method described in Section 2.4, under the optimized gradient elution conditions established in Section 2.5.

2.11. Statistical Analysis

Data are expressed as Mean ± SD unless otherwise stated. Baseline serum concentrations of vitamin C among the four independent clinical groups (Table 6) were compared using one-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) post-hoc test for pairwise multiple comparisons. A p-value < 0.05 was considered statistically significant, and $p < 0.0001$ was reported where applicable. Longitudinal changes in analyte concentrations between baseline (Day 1-3) and post-detoxification (Day 60) within each addicted group (Table 7) were evaluated using the paired-samples t-test.

3. Results and Discussion

3.1. Optimization of Chromatographic Conditions.

Serial systematic experiments were conducted to establish a rapid, simple, and highly efficient reversed-phase HPLC method for the simultaneous separation and quantitative determination of amphetamine, ephedrine, and vitamin C. Chromatographic separations were performed on a Hybrid C18 column (250 mm × 4.6 mm, 5 µm) maintained at ambient temperature, utilizing multi-wavelength PDA detection monitored at 260 nm, 270 nm, and 280 nm for amphetamine, ephedrine, and vitamin C, respectively. The mobile phase volumetric ratio (50:50 to 90:10, v/v) was initially screened, at a flow rate of 0.7 mL/min, to evaluate its impact on eluent strength, solute retention kinetics, and detector sensitivity. As the organic modifier proportion varied, peak area and shape shifted significantly. Optimal detector responses and favorable peak were using ratios: 80:20 v/v of acetonitrile: methanol with orthophosphoric acid for amphetamine (975.08 mAU·s), 90:10 v/v of acetonitrile: water with 0.1% formic acid and 5 mM ammonium formate for ephedrine (865.08 mAU·s), and 80:20 v/v of methanol: phosphate buffer with tetrabutylammonium, TBA for Ascorbic acid (422.14 mAU·s). The inclusion of TBA supports in situ ion-pair complexation with the strongly polar ascorbic acid, enhancing hydrophobic retention [29, 30]. The effect of mobile phase pH at 4.0, 7.0, and 9.0 was studied at the optimized mobile phase ratios and a flow rate of 0.7 mL. min^{-1} to meet the conflicting ionization requirements of the target compounds. The acidic nature of ascorbic acid versus the basic amine nature of amphetamine and ephedrine gave a specific analytical challenge. At a pH of 9.0, ionization of the basic drugs was inhibited, conserving them in their

unionized free base forms in basic media; this decreased the secondary silanol interactions on the silica column backbone, leading to maximized detector sensitivity (1254.14 mAU·s for amphetamine and 978.08 mAU·s for ephedrine) [25,26]. An acidic pH of 4.0 was required for investigating ascorbic acid to keep its nonionized state, causing its retention stability and a good peak area response (569.08 mAU·s) [27,28]. The flow rate was determined by testing 0.5 to 1.5 mL·min^{-1}, at the optimized mobile phase ratios and pH to prove the optimal balance between analysis run time, backpressure of the column, and efficiency of theoretical plates. While a lower flow rate of 0.5 mL·min^{-1} yielded higher peak areas for Ephedrine (1008.77 mAU·s) and ascorbic acid (588.07 mAU·s), a flow rate of 0.7 mL·min^{-1} was approved as optimal, providing the highest peak area for Amphetamine (1254.14 mAU·s), peak symmetry, and stable backpressure of the column. Combining these optimized variables into a time-programmed gradient elution profile enabled complete baseline resolution (Rs = 4.04 for Amp/Eph and Rs = 2.15 for Eph/Vit C) with high peak symmetry and reproducible retention times (3.22 min for Amphetamine at pH 9.0, 5.78 min for Ephedrine at pH 9.0, and 7.25 min for ascorbic acid at pH 4.0), within a total run time of 14 min, inclusive of column re-equilibration. The representative HPLC chromatogram of the optimized separation and the corresponding system suitability metrics are presented in **Fig 2.**

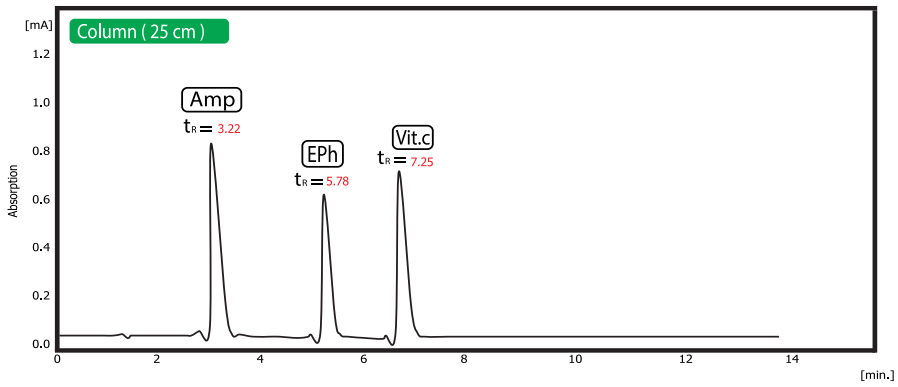


Fig 2. Representative HPLC-PDA chromatogram of the simultaneous separation of amphetamine, ephedrine, and ascorbic acid (Vit. C) (5 ng. mL^{-1} each) under the optimized gradient elution conditions, showing baseline resolution and detection at 260, 270, and 280 nm, respectively.

3.2. Chromatographic Functions and Column Efficiency

The efficiency and chromatographic performance of the developed HPLC method were evaluated under the optimized gradient elution conditions using a Hybrid C18 column (250 mm × 4.6 mm, 5 µm). Chromatographic parameters such as retention time (t_R), capacity factor (k′), theoretical plates (N), effective theoretical plates (N_{eff}), and height equivalent to an effective theoretical plate (H_{eff}) were investigated and calculated for each substance under study, as presented in Table 1, while selectivity (α), baseline resolution (Rs), and required theoretical plates (N_{req}) were studied and calculated for each pair of peaks, as shown in Table 2, to confirm system suitability and assess robustness. The dead time (t_0) was 1.60 min at a linear velocity (u) of 156.25 mm.min^{-1}, improving the mass transfer

efficiency of the mobile phase by the stationary phase. High chromatographic selectivity was obtained between neighboring peak pairs, with selectivity factors (α) of 2.58 for amphetamine and ephedrine and 1.35 for ephedrine and ascorbic acid, which give a clear chemical identification between the three substances. Excellent baseline resolution was achieved, Rs = 4.04 for the first neighboring peak pair (Amp and Eph) and Rs = 2.15 for the second neighboring peak pair (Eph and ascorbic acid), meeting standard criteria for substances, which is Rs > 1.5 [29,30]. Column efficiency was confirmed by the theoretical plate counts (N), at 705 for amphetamine, 1542 for ephedrine, and 1388 for ascorbic acid. lowering of the plate count for amphetamine is referred to as initial zone broadening near t0, whereas the last eluting analytes showed an increase in column efficiency [31,32], with low effective plate heights were H_{eff} = 0.031 cm for ephedrine and 0.030 cm for ascorbic acid. The effective theoretical plates were Neff = 806 for Ephedrine and 843 for ascorbic acid and were below the theoretically required plate count (N_{req} = 876); that is, the actual baseline resolution obtained was Rs = 2.15, and it still met the acceptance criterion, which is Rs > 1.5, indicating a good separation, with a narrower than the theoretical plate requirement under changing routine operational or column aging [39].

Table 1. Column efficiency parameters for amphetamine, ephedrine, and ascorbic acid on the Hybrid C18 column (250 mm × 4.6 mm, 5 µm); t_0 = 1.60 min. Capacity factor (k′) calculated.

Analyte	t_R (min)	k′	N	N_{eff}	H_{eff} (cm)
Amphetamine	3.22	1.013	705	178	0.140
Ephedrine	5.78	2.610	1542	806	0.031
ascorbic acid	7.25	3.530	1388	843	0.030

Table 2. Selectivity (α), resolution (Rs), and required theoretical plates (N_{req}) between neighboring peak pairs.

Neighboring Peak pair	α	Rs	N_{req}
Amphetamine / Ephedrine	2.58	4.04	184
Ephedrine / ascorbic acid	1.35	2.15	876

3.3. Method Validation

3.3.1. Linearity and Dynamic Range

Under the optimized conditions for the chromatographic method, linearity was determined by calibration curves for amphetamine, ephedrine, and ascorbic acid using peak area responses across a range of 5.0-25.0 ng. mL^{-1}. Linear regression analysis showed a good proportional relationship between the concentration of the substance and detector response for all analytes, as illustrated in Figure 3.

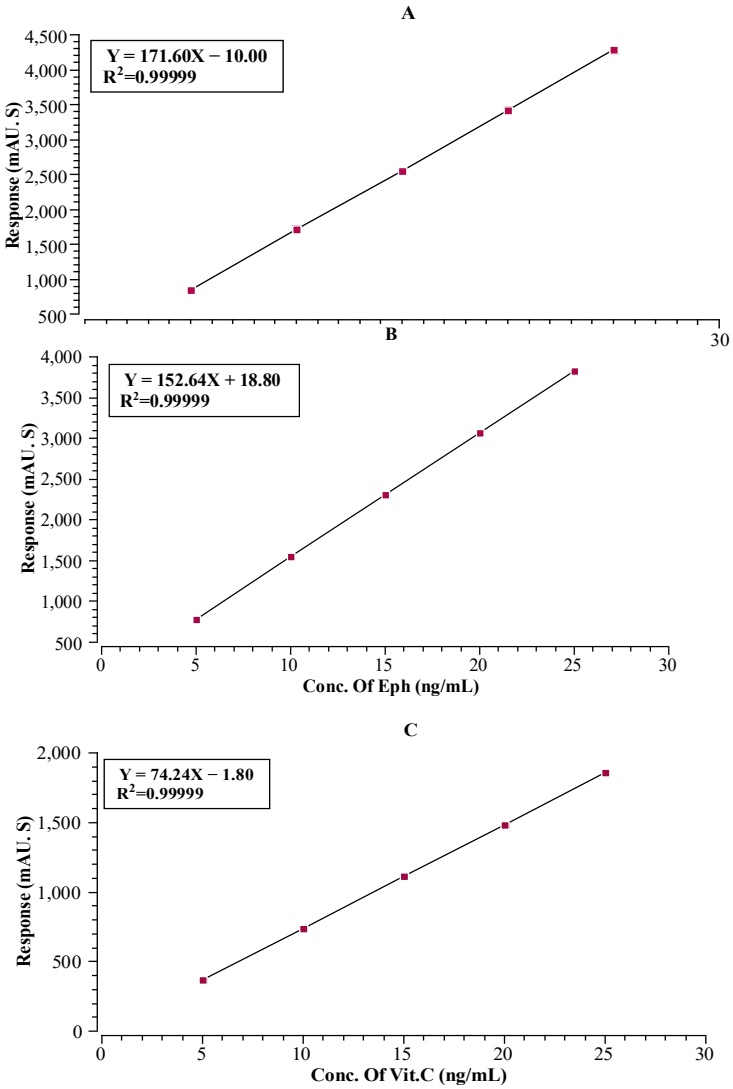


Fig 3. Calibration curves of (A) Amphetamine, (B) Ephedrine, and (C) ascorbic acid (Vit. C), showing peak area versus nominal concentration over the range of 5.0-25.0 ng/mL (n = 3 at each level), with the corresponding linear regression equations and coefficients of determination (R^2).

Table 3 showed that the high precision of the linear model was confirmed by the maximum coefficient of determination that was $R^2 = 0.99999$ for all three substances. The calculated 95% confidence intervals for slopes and intercepts further validate the method's reliability; since all confidence intervals for the intercepts contained zero,

which were -43.92, 23.92 for Amp, and −6.53, 44.13 for Eph, then −10.50, 6.90for ascorbic acid. The absence of constant systematic errors across the calibration range was demonstrated. Mandel's fitting test was conducted to statistically evaluate straight-line linearity versus non-linear quadratic [34,35]. The calculated F-values were Fcalc = 2.72, 3.91, and 0.03 for Amp, Eph, and ascorbic acid, respectively, and were substantially less than the critical tabular value, which was F = 18.51 at α = 0.05, f1 = 1, f2 = 2, improving that the linear model is statistically appropriate. Furthermore, the relative standard deviation of the response factors (%RSD of RF) values was 0.35% for Amp, 0.48% for Eph, and 0.16% for ascorbic acid, well within the acceptance limit (≤ 2.5%) [42].

3.3.2. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Method sensitivity was studied by determining the limit of detection (LOD) and limit of quantitation (LOQ) for each substance, calculated using the standard error of the intercept (Sa, representing σ) and the slope (b, representing S) from the calibration data, using the equations (LOD = 3.3σ/S and LOQ = 10σ/S) [43]. As shown in Table 3, the calculated LOD values were 0.195 ng/mL for amphetamine, 0.164 ng/mL for ephedrine, and 0.116 ng/mL for ascorbic acid. The LOQ values were 0.593 ng. mL^{-1}, 0.497 ng. mL^{-1}, and 0.352 ng. mL^{-1} for amphetamine, ephedrine, and ascorbic acid, respectively. These low limits indicated the high sensitivity of the RP-HPLC method, making it reliable for trace-level detection and accurate quantitative analysis in routine quality control.

Table 3. Statistical parameters for the linear regression, LOD, and LOQ of amphetamine, ephedrine, and ascorbic acid.

Statistical Parameter	Amphetamine	Ephedrine	Ascorbic acid
Linearity range (ng/mL)	5.0–25.0	5.0–25.0	5.0–25.0
Regression equation	Y = 171.60X − 10.00	Y = 152.64X + 18.80	Y = 74.24X − 1.80
95% CI of slope	[169.55, 173.65]	[151.11, 154.17]	[73.72, 74.76]
95% CI of intercept	[−43.92, 23.92]	[−6.53, 44.13]	[−10.50, 6.90]
Coefficient of determination (R^2)	0.99999	0.99999	0.99999
Sy/x	10.17	7.59	2.61
% y-intercept	−0.23%	+0.49%	−0.10%
%RSD of response factor	0.35%	0.48%	0.16%
Mandel's F-test	2.72	3.91	0.03
LOD (ng/mL)	0.195	0.164	0.116
LOQ (ng/mL)	0.593	0.497	0.352
Relative response factor (RRF)	1.00 (Ref.)	0.889	0.433

3.3.3. Accuracy and Precision

Method accuracy and precision were determined by analyzing Amphetamine, Ephedrine, and ascorbic acid at four different concentrations (7.0, 12.0, 17.0, and 23.0 ng. mL^{-1}). Intra-day precision, or repeatability, was evaluated by triplicate analysis (n = 3) within the same day, while inter-day precision, which means intermediate precision, was evaluated by triplicate analysis (n = 3) on three different days. Accuracy was assessed as percentage recovery of the found concentration relative to the first concentration, and precision was assessed as the relative standard deviation (%RSD) of the replicate measurements. Results are illustrated in Tables 4 and 5. Intra-day recovery values ranged from 99.71% to 100.28% for all substances, with a %RSD within 0.24%, confirming excellent repeatability within a single analytical run time (Table 4). Inter-day recovery values were from 99.73% to 100.08%, with %RSD ranging from 0.68% to 0.90% as shown in Table 5; while modestly higher than the intra-day values, as expected due to day-to-day variability in mobile phase preparation, column efficiency, and instrument response, all values kept within the acceptable precision limit (%RSD ≤ 2%) recommended by ICH Q2(R2) guidelines [44], [45]. The expanded uncertainty (U%, k = 2) did not exceed ±1.80% under inter-day conditions, which proves the robustness and reproducibility of the developed method.

Table 4. Intra-day accuracy and precision data for amphetamine, ephedrine, and ascorbic acid (n = 3).

Analyte	Nominal Conc. (ng/mL)	Found Conc. (Mean ± SD, ng/mL)	%Recovery ± %RSD	Expanded Uncertainty (U%, k=2)
Amp	7.0	6.990 ± 0.017	99.85% ± 0.24%	±0.48%
	12.0	12.031 ± 0.016	100.26% ± 0.13%	±0.26%
	17.0	16.991 ± 0.016	99.95% ± 0.09%	±0.18%
	23.0	23.038 ± 0.039	100.17% ± 0.17%	±0.34%
Eph	7.0	6.980 ± 0.012	99.71% ± 0.17%	±0.34%
	12.0	12.033 ± 0.015	100.28% ± 0.13%	±0.26%
	17.0	16.968 ± 0.021	99.81% ± 0.12%	±0.24%
	23.0	23.053 ± 0.031	100.23% ± 0.13%	±0.26%
Vit. C	7.0	7.008 ± 0.016	100.11% ± 0.23%	±0.46%
	12.0	11.979 ± 0.017	99.83% ± 0.14%	±0.28%
	17.0	17.023 ± 0.025	100.14% ± 0.15%	±0.30%
	23.0	22.957 ± 0.024	99.81% ± 0.10%	±0.20%

Table 5. Inter-day accuracy and precision data for amphetamine, ephedrine, and ascorbic acid (n = 3, three consecutive days).

Analyte	Nominal Conc. (ng/mL)	Found Conc. (Mean ± SD, ng/mL)	%Recovery ± %RSD	Expanded Uncertainty (U%, k=2)
Amp	7.0	6.981 ± 0.062	99.73% ± 0.90%	±1.80%
	12.0	12.006 ± 0.090	100.05% ± 0.75%	±1.50%
	17.0	16.978 ± 0.115	99.87% ± 0.68%	±1.36%
	23.0	23.003 ± 0.172	100.01% ± 0.75%	±1.50%
Eph	7.0	6.981 ± 0.054	99.73% ± 0.76%	±1.52%
	12.0	12.009 ± 0.089	100.08% ± 0.73%	±1.46%
	17.0	16.962 ± 0.122	99.78% ± 0.71%	±1.42%
	23.0	23.009 ± 0.180	100.04% ± 0.78%	±1.56%
Vit. C	7.0	7.002 ± 0.059	100.03% ± 0.85%	±1.70%
	12.0	11.974 ± 0.100	99.78% ± 0.84%	±1.68%
	17.0	17.010 ± 0.139	100.06% ± 0.82%	±1.64%
	23.0	22.943 ± 0.188	99.75% ± 0.82%	±1.64%

3.4. Clinical Application

3.4.1. Serum Concentration Profiles

Table 6 summarizes mean serum concentrations of amphetamine, ephedrine, and ascorbic acid for each group. Amphetamine and ephedrine were not detected (UDL, under the respective limit of detection) in groups without addiction, improving the analytical specificity of the developed method in a biological matrix. Serum ascorbic acid concentrations were lower in all addicted groups (G II–IV) relative to the healthy control group, with increased antioxidant reserves under oxidative stress related to psychostimulant exposure. The decreased serum ascorbic acid concentrations in the drug addiction groups (3526±110.94 ng/mL ng. mL^{-1} in amphetamine users and 3173±241.77 ng/mL in co-users, versus 8718±354.04 ng/mL in the control group; $P < 0.0001$) can be related to biochemical reactions. Amphetamine and ephedrine strongly stimulate toxic neuronal catecholamine pathways, leading to systemic oxidative stress and increased accumulation of reactive oxygen species (ROS). Ascorbic acid, a primary, water-soluble, and endogenously available antioxidant, is rapidly consumed to sweep ROS and neutralize toxic dopamine quinones. Ascorbic acid acts as a nonexchangeable electron donor to dopamine beta-hydroxylase during catecholamine synthesis, leading to chronic sympathetic stimulation and depleting functional ascorbic acid stores. These biochemical burdens, in addition to drug-induced loss of appetite and altered gastrointestinal absorption, confirm the physiological deficit [46,47].

Table 6. Mean ± SD serum concentrations **(ng/mL)** of Amphetamine, Ephedrine, and Ascorbic acid at baseline across the four clinical study groups (N = 100, n = 25 per group).

Clinical Group	Amphetamine (Mean±SD)	Ephedrine (Mean±SD)	Ascorbic acid (Mean±SD)
G. I: Healthy Control	[a] UDL	[a] UDL	8718±354.04
G. II: Amphetamine addicted	293.43±26.60	[a] UDL	3526±110.94
G. III: Ephedrine-Addicted (Illicit Non-medical Use)	[a] UDL	281.23±3.56	3978±120.62
G. IV: Amphetamine, Ephedrine addicted	381.45±32.00	214.59±1.43	3173±241.77

[a] UDL = Under Detection Limit. Values are presented as Mean ± SD. All addicted participants (Groups II–IV) were recruited from an addiction treatment hospital with a confirmed clinical diagnosis and received antioxidant supplementation; serum Ascorbic acid was the primary outcome measure. Group III participants were athletes whose ephedrine dependence originated from illicit, non-medical performance-enhancing use.

3.4.2. Longitudinal Changes Following Detoxification

Serum concentrations of amphetamine, ephedrine, and Ascorbic acid were re-evaluated in Groups II–IV following the 60-day detoxification period as shown in Table7. Amphetamine and ephedrine concentrations decreased from baseline to near-trace levels approaching the limits of detection for all addicted groups (G II-IV), in harmony with body clearance of both stimulants, amphetamine and ephedrine, over the detoxicant period. Serum ascorbic acid concentrations recovered toward the range observed in the healthy control group, as shown in Table 6, improving the hypothesis that ascorbic acid levels reflected antioxidant depletion secondary to psychostimulant use related to oxidative stress, rather than a direct drug interaction [46,47]

Table 7. Serum concentrations **(ng/mL)** of amphetamine, ephedrine, and Ascorbic acid in Groups II-IV on Day 60 compared with baseline **(Table 6)**. Group I (healthy control) was evaluated at baseline only.

Clinical Group (Day60)	Amphetamine (Mean±SD)	Ephedrine (Mean±SD)	Ascorbic acid (Mean±SD)	Recovery & Significance Status
G. II: Amphetamine-Addicted Recovery	2.18 ± 0.84[a]	[D] UDL	9678.01 ±245.21[b]	Significant clearance (P<0.0001) [c]
G. III: Ephedrine-Addicted (Athletes; Illicit Non-medical Use) Recovery	[D] UDL	1.48 ± 0.35[a]	10534.10 ±434.68[b]	Significant clearance (P<0.0001) [c]
G. IV: Amphetamine + Ephedrine Co-Addicted Recovery	3.42 ± 0.39[a]	0.99 ± 0.19[a]	6454.85 ±152.33[b]	Significant clearance (P<0.0001) [c]

[a] significant reduction in drug concentration relative to baseline (Day 1). [b] Significant increase in serum Ascorbic acid relative to baseline (Day 1). [c] Paired comparison vs. Day 1 baseline within the same group (P<0.0001). [D] UDL = Under Detection Limit**.**

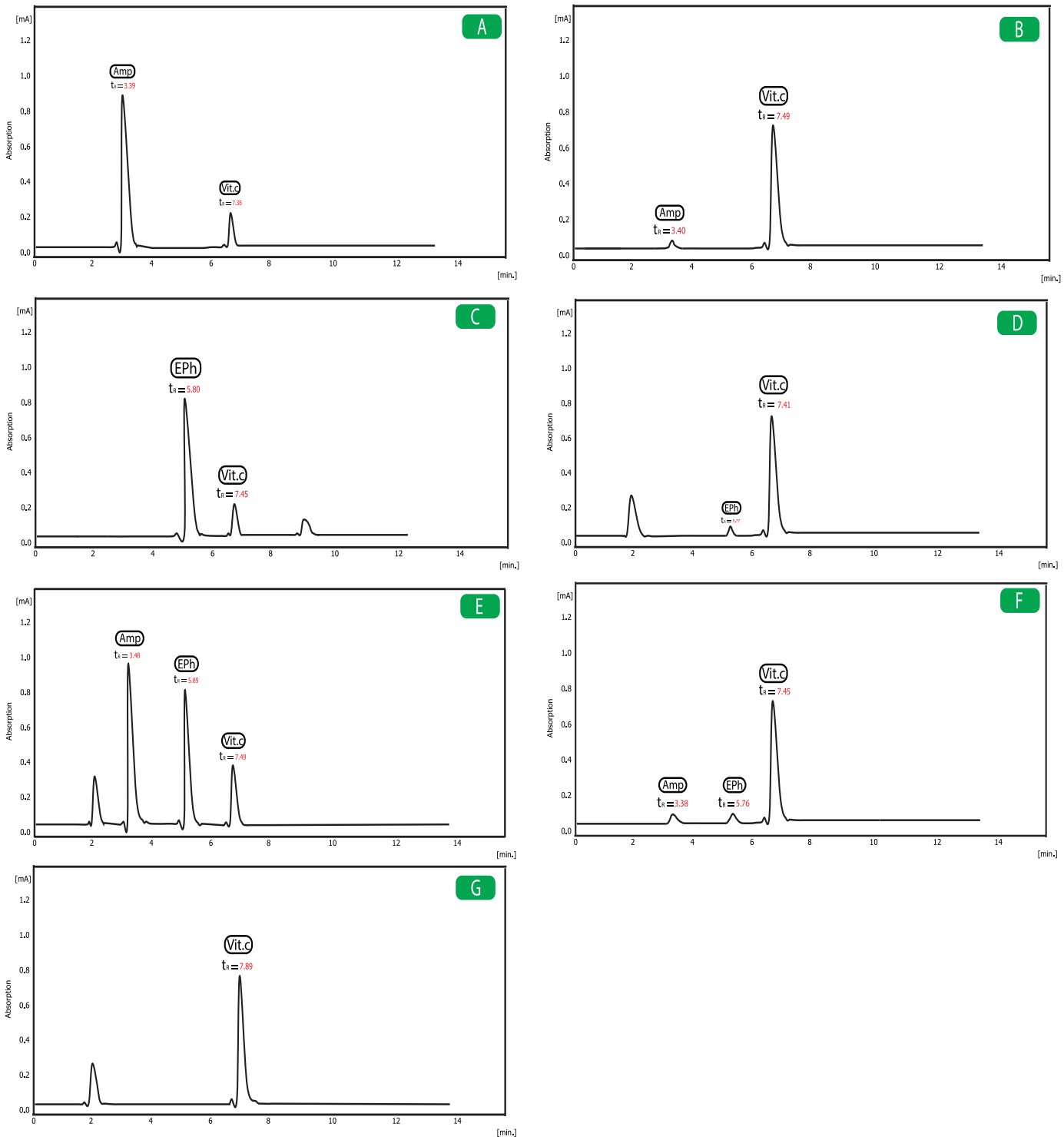


Fig 4. Representative HPLC-PDA Serum Chromatograms Across Clinical Cohorts Before and After detoxicant period.

3.4.3. Artificial Neural Network (ANN) Modeling

The error histogram, which has 20 bins, indicated the distribution of prediction errors, which are defined as Targets minus Outputs for the developed 3-10-3 artificial neural network model. As illustrated in the histogram, the vast majority of the data are densely clustered around the zero-error line that improved by the vertical orange line. This close distribution around zero indicated that the prediction errors are negligible and randomly distributed, implying the absence of any systematic bias and proving the high accuracy and reliability of the model as illustrated in Fig. 5A. The predictive efficiency and generalization capability of the optimized neural network model were further assessed through linear regression analyses between the target and output values across training, validation, and test datasets. The high correlation coefficients (R-values) obtained, specifically 0.92521 for training, 0.97507 for validation, and an exceptional 0.98682 for testing, exhibited a strong linear agreement between the experimental and predicted responses. These high R-values prove that the 3-10-3 ANN architecture mapped the complex non-linear relationships of the chromatographic condition, confirming its high robustness and suitability for predictive optimization, as illustrated in Fig. 5B.

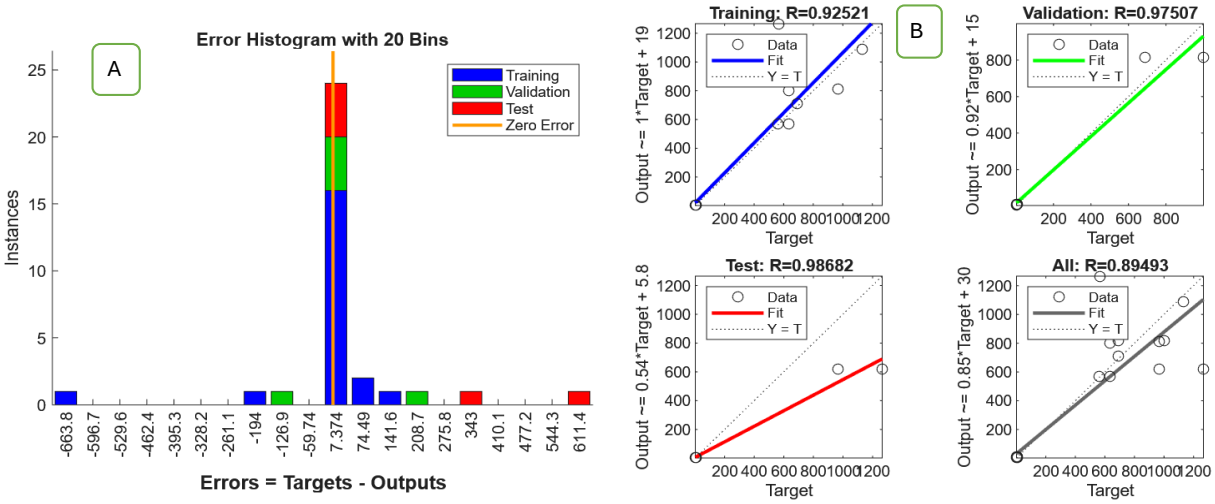


Fig 5. Performance evaluation of the optimized 3-10-3 artificial neural network model: (A) Error histogram with 20 bins illustrating the prediction error distribution, and (B) Linear regression analyses (R-values) between target and output values across training, validation, test, and all datasets.

To make a robust predictive mapping of the chromatographic attitude, a multi-layer feedforward artificial neural network (ANN) featuring a 3-10-3 topology was developed and implemented using MATLAB, as shown in Figure. 6A. As structurally illustrated in Figure 6B, the network effectively links the three operational input parameters, such as mobile phase ratio, pH, and Flow Rate, to a hidden layer comprising ten processing neurons equipped with weight matrices (W), bias vectors (b), and nonlinear transfer functions designed to resolve interactions. The information is directed through the output layer to determine the three analytical parameters, such as Retention Time, Peak Area, and Tailing Factor

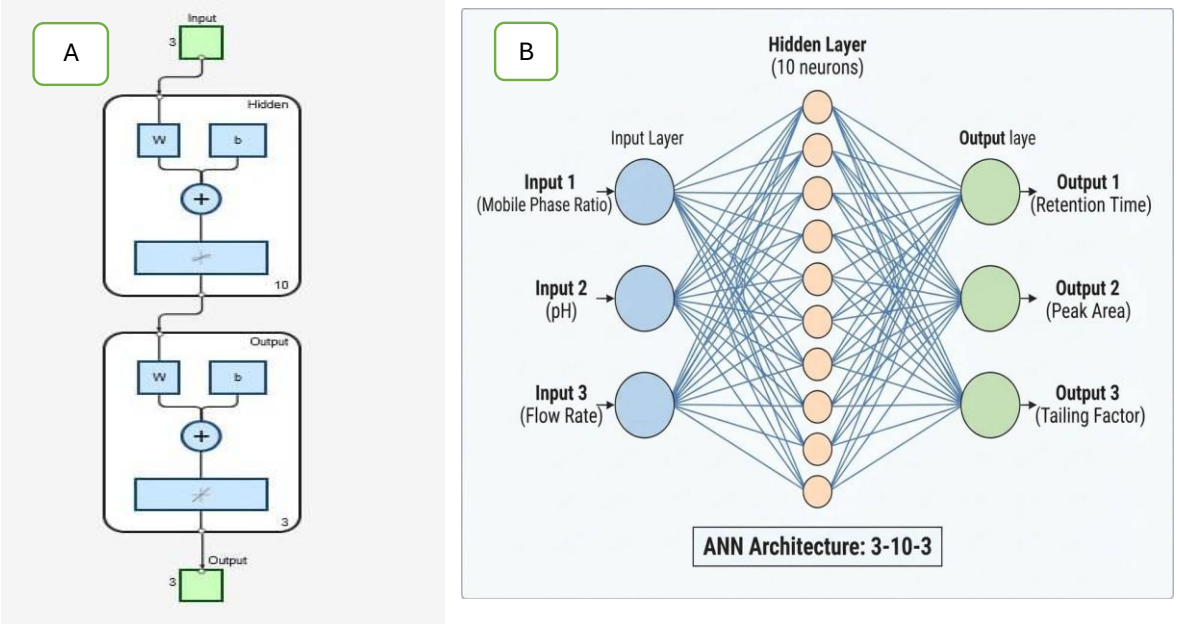


Fig 6. Topological and architectural configuration of the optimized 3-10-3 artificial neural network model: (A) Internal MATLAB block diagram illustrating the layers, weight matrices (W), bias vectors (b), and transfer functions, and (B) Schematic representation linking the chromatographic operational inputs to the analytical output responses.

Conclusion

In this study, a high-performance liquid chromatography (RP-HPLC) method was successfully developed and validated for the simultaneous, rapid, highly sensitive determination of amphetamine, ephedrine, and ascorbic acid concentrations in human serum using a gradient elution mode. Validation of the statistical model was performed according to ICH guidelines, indicating excellent linearity ($R^2 > 0.999$), high accuracy, and robustness, with limit of detection (LOD) values below 0.20 ng/mL for all analytes, and the standardized unit of ng/mL enabled accurate analytical comparisons between calibration standards and human serum samples, providing a validated analytical tool for both forensic and clinical toxicology applications. Mapping of the chromatographic attitude, a multi-layer feedforward artificial neural network (ANN) featuring a 3-10-3 topology, was developed and implemented using MATLAB. The network effectively links the three operational input parameters, such as mobile phase ratio, pH, and Flow Rate. The information is directed through the output layer to determine the three analytical parameters, such as Retention Time, Peak Area, and Tailing Factor.

References.

[1] A. I. Al-Asmari, "A critical review of workplace drug testing methods for old and new psychoactive substances: gaps, advances, and perspectives," Saudi Pharmaceutical Journal, 2024. [Online]. Available: https://www.sciencedirect.com/science/article/pii/S1319016424001154

[2] V. Battini et al., "Psychiatric and non-psychiatric drugs causing false-positive amphetamines urine test in psychiatric patients: a pharmacovigilance analysis using FAERS", doi: 10.6084/M9.FIGSHARE.23095539.V1.

[3] J. D. Pope, O. H. Drummer, and H. G. Schneider, "False-positive amphetamines in urine drug screens: a 6-year review," Journal of Analytical Toxicology, 2023. [Online]. Available: https://academic.oup.com/jat/article-abstract/47/3/263/6823803

[4] "Blurred Lines: The Ethical Pitfalls of Urine Drug Screens - EMOttawa Blog." Accessed: Aug. 04, 2026. [Online]. Available: https://emottawablog.com/2024/11/the-issue-with-the-urine-tox-screen/

[5] A. Saitman, R. L. Fitzgerald, K. Lund, R. T. Suhandynata, and M. Menlyadiev, "False positive urine drug screens," J. Anal. Toxicol., vol. 50, no. 4, May 2026, doi: 10.1093/JAT/BKAG007.

[6] N. Sainz-Pastor et al., "Challenges in the interpretation of amphetamine results: differences of reactivity between immunoassay and immunochromatography," Scand. J. Clin. Lab. Invest., 2026, doi: 10.1080/00365513.2026.2694040.

[7] J. Y. Chen, G. Y. Chen, L. M. Wu, C. H. Kuo, and T. I. Weng, "Impurities in over-the-counter pseudoephedrine leading to methcathinone detection in urine," Forensic Sci. Int., vol. 356, p. 111964, Mar. 2024, doi: 10.1016/J.FORSCIINT.2024.111964.

[8] T. Willeman et al., "Underreporting of synthetic cathinone poisoning with clinical immunoassays: An experimental and observational study," Ann. Clin. Biochem., vol. 62, no. 6, pp. 440–446, Nov. 2025, doi: 10.1177/00045632251331404;PAGE:STRING:ARTICLE/CHAPTER.

[9] O. Joseph, M. Eberle, and M. Lieberman, "Metabolites in Urine that Interfere with the Sandell-Kolthoff Assay for Urinary Iodine," Biological Trace Element Research 2023 202:2, vol. 202, no. 2, pp. 466–472, May 2023, doi: 10.1007/S12011-023-03710-3.

[10] M. Abdelati, M. Hilal, K. Mohamed, and R. Elsayed, "Review on the effects of adulterants on drug abuse testing in urine samples," Ain Shams Journal of Forensic Medicine and Clinical Toxicology, 2020. [Online]. Available: https://journals.ekb.eg/article_102277.html

[11] A. Jangra, H. Khajuria, B. Prakash Nayak, K. Goyal, and R. K. Sarin, "Implementation of Modified D-SPE Method for Simultaneous Quantification of Methamphetamine & Mephedrone Using LC-MS/MS," 2024, doi: 10.33263/LIANBS132.100.

[12] M. Di Rago et al., “High Throughput Detection of 327 Drugs in Blood by LC–MS-MS with Automated Data Processing,” J. Anal. Toxicol., vol. 45, no. 2, pp. 154–183, Feb. 2021, doi: 10.1093/JAT/BKAA057.

[13] J. Bickel et al., “Chiral analysis of amphetamine, methamphetamine, MDMA and MDA enantiomers in human hair samples,” J. Anal. Toxicol., vol. 48, no. 4, pp. 226–234, May 2024, doi: 10.1093/JAT/BKAE026.

[14] R. Boscolo-Berto, “Challenges and future trends of forensic toxicology to keep a cut above the rest,” ADVANCES IN CLINICAL AND EXPERIMENTAL MEDICINE, vol. 33, no. 5, pp. 423–425, May 2024, doi: 10.17219/ACEM/185730.

[15] S. Debnath, M. Das, S. Mondal, B. K. Sarkar, and G. Babu, “Advances in chromatography: contemporary techniques and applications,” Essential Chem, vol. 2, no. 1, pp. 1–27, Dec. 2025, doi: 10.1080/28378083.2025.2466624.

[16] J. A. Chenoweth, C. K. LaValley, C. S. Eberhardt, D. K. Colby, T. E. Albertson, and N. K. Tran, “Cost-effective drug testing: analytical methodology, best practices, and clinical utility,” The Journal of Applied Laboratory Medicine, 2025. [Online]. Available: https://academic.oup.com/jalm/article-abstract/10/1/92/7931429

[17] A. Miłos, M. Gackowski, A. Przybylska, P. Kośliński, and M. Koba, “Comparison of sensitivity and specificity of commercial amphetamine tests,” AppliedChem, 2023. [Online]. Available: https://www.mdpi.com/2673-9623/3/1/10

[18] “VALIDATION OF ANALYTICAL PROCEDURES Q2(R2)”.

[19] P. Araujo, “Key aspects of analytical method validation and linearity evaluation,” Journal of Chromatography B, vol. 877, pp. 2224–2234, 2009, doi: 10.1016/j.jchromb.2008.09.030.

[20] F. Raposo and C. Ibelli-Bianco, “Performance parameters for analytical method validation: Controversies and discrepancies among numerous guidelines,” TrAC Trends in Analytical Chemistry, vol. 129, p. 115913, Aug. 2020, doi: 10.1016/J.TRAC.2020.115913.

[21] F. T. Peters, “Principles of Forensic Toxicology, 5th edition (2020),” J. Anal. Toxicol., vol. 45, no. 4, pp. e6–e7, Apr. 2021, doi: 10.1093/JAT/BKAB020.

[22] F. T. Peters, J. ; M. Hartung, H. / Saar, ; M Herbold, and G. Schmitt, “APPENDIX B To the GTFCh Guidelines for quality assurance in forensic-toxicological analyses Requirements for the validation of analytical methods,” 2009.

[23] A. Aisa-Álvarez et al., “Randomized Clinical Trial of Antioxidant Therapy Patients with Septic Shock and Organ Dysfunction in the ICU: SOFA Score Reduction by Improvement of the Enzymatic and Non-Enzymatic Antioxidant System,” Cells, vol. 12, no. 9, May 2023, doi: 10.3390/CELLS12091330.

[24] I. Pérez-Torres et al., “Impact of Treatment with Antioxidants as an Adjuvant to Standard Therapy in Patients with Septic Shock: Analysis of the Correlation between Cytokine Storm and Oxidative Stress and Therapeutic Effects,” Int. J. Mol. Sci., vol. 24, no. 23, Dec. 2023, doi: 10.3390/IJMS242316610.

[25] A. Podkowińska and D. Formanowicz, “Chronic kidney disease as oxidative stress-and inflammatory-mediated cardiovascular disease,” Antioxidants, vol. 9, no. 8, pp. 1–54, Aug. 2020, doi: 10.3390/ANTIOX9080752.

[26] K. E. Chan et al., “Longitudinal Outcomes Associated With Metabolic Dysfunction-Associated Steatotic Liver Disease: A Meta-analysis of 129 Studies,” Clinical Gastroenterology and Hepatology, vol. 22, no. 3, pp. 488-498.e14, Mar. 2024, doi: 10.1016/J.CGH.2023.09.018.

[27] M. K. Tuck, D. W. Chan, D. Chia, A. K. Godwin, W. E. Grizzle, K. E. Krueger, W. Rom, and M. Sanda, “Standard operating procedures for serum and plasma collection: Early Detection Research Network consensus statement standard operating procedure integration,” Journal of Proteome Research, 2009. [Online]. Available: https://pmc.ncbi.nlm.nih.gov/articles/PMC2655764/

[28] D. M. Boland, “Disposition of Toxic Drugs and Chemicals in Man, 12th Edition,” J. Anal. Toxicol., vol. 44, no. 9, pp. e13–e13, Jan. 2021, doi: 10.1093/JAT/BKAA121.

[29] A. Khrisanfova, M. Smagina, G. Maksimov, G. Tsizin, O. Shpigun, and A. Chernobrovkina, “Evaluating independent effect of mobile phase components on retention mechanism of ionizable analytes in hydrophilic interaction liquid chromatography,” Journal of Chromatography A, vol. 1758, Sep. 2025, doi: 10.1016/J.CHROMA.2025.466201.

[30] R. Walther, O. Scherf-Clavel, and U. Holzgrabe, “Method transfer from ion pair chromatography to charged aerosol detector-compatible mixed-mode chromatography: A case study using carbocisteine,” Journal of Chromatography Open, vol. 1, Nov. 2021, doi: 10.1016/J.JCOA.2021.100014.

[31] G. S. Maksimov, A. O. Shemiakina, A. S. Uzhel, and A. V. Chernobrovkina, “Изучение хроматографических свойств разработанного гиперразветвленного цвиттер-ионного сорбента на основе силикагеля в режиме гидрофильной хроматографии,” Сорбционные и хроматографические процессы, vol. 24, no. 3, pp. 304–320, Jul. 2024, doi: 10.17308/SORPCHROM.2024.24/12234.

[32] D. V. McCalley, “Is hydrophilic interaction chromatography with silica columns a viable alternative to reversed-phase liquid chromatography for the analysis of ionisable compounds?,” Journal of Chromatography A, vol. 1171, no. 1–2, pp. 46–55, Nov. 2007, doi: 10.1016/J.CHROMA.2007.09.047.

[33] E. Jeney-Nagymate and P. Fodor, “The stability of vitamin C in different beverages,” British Food Journal, vol. 110, no. 3, pp. 296–309, 2008, doi: 10.1108/00070700810858709.

[34] L. Shaik and S. Chakraborty, “Ultrasound processing of sweet lime juice: Effect of matrix pH on microbial inactivation, enzyme stability, and bioactive retention,” Journal of Food Process Engineering, vol. 46, no. 6, Jun. 2023, doi: 10.1111/JFPE.14231.

[35] P. A. Makoni, M. T. R. Chikukwa, S. M. M. Khamanga, and R. B. Walker, “Stability indicating HPLC-ECD method for the analysis of clarithromycin in pharmaceutical dosage forms: method scaling versus re-validation,” Scientia Pharmaceutica, vol. 87, no. 4, Dec. 2019, doi: 10.3390/SCIPHARM87040031.

[36] M. Abualhasan, M. Mousa, H. Izhiman, M. Alkhatib, and I. Aghbar, “A High-Performance Liquid Chromatography Method for Simultaneous Analysis of Naringin, Ascorbic Acid, and Caffeine: Design, Development, and Validation,” International Journal of Analytical Chemistry, vol. 2026, no. 1, 2026, doi: 10.1155/IANC/6287324.

[37] J. J. Baeza-Baeza and M. C. García-Álvarez-Coque, “A theoretical plate model accounting for slow kinetics in chromatographic elution,” Journal of Chromatography A, vol. 1218, no. 31, pp. 5166–5174, Aug. 2011, doi: 10.1016/J.CHROMA.2011.05.086.

[38] N. Kovalenko and C. F. Landes, “The Impact of Rare Adsorption Site Clustering on Peak Broadening in Chromatography,” Analytical Chemistry, vol. 97, no. 37, pp. 20237–20246, Sep. 2025, doi: 10.1021/ACS.ANALCHEM.5C03108.

[39] S. D. Selaya, N. Abrigo, C. Jones, M. Korang-Yeboah, P. J. Faustino, and D. Shakleya, “Development and Validation of a Headspace GC–MS Method for Simultaneous Quantification of Antimicrobial Preservatives in Biopharmaceutical Peptide Formulations,” Biomedical Chromatography, vol. 39, no. 1, Jan. 2025, doi: 10.1002/BMC.6045.

[40] F. Raposo and D. Barceló, “Assessment of goodness-of-fit for the main analytical calibration models: Guidelines and case studies,” TrAC - Trends in Analytical Chemistry, vol. 143, Oct. 2021, doi: 10.1016/J.TRAC.2021.116373.

[41] R. I. Rawski, P. T. Sanecki, K. M. Kijowska, P. M. Skitał, and D. E. Saletnik, “Regression analysis in analytical chemistry. Determination and validation of linear and quadratic regression dependencies,” South African Journal of Chemistry, vol. 69, pp. 166–173, 2016, doi: 10.17159/0379-4350/2016/V69A20.

[42] Umme Kulsum and Naveen Kumar G S, “A Review of Analytical Methods for Quantitative Determination and Validation of Fluvoxamine,” Journal of Pharma Insights and Research, vol. 3, no. 2, pp. 105–112, Apr. 2025, doi: 10.69613/937YA379.

[43] M. Taleuzzaman, “Limit of Blank (LOB), Limit of Detection (LOD), and Limit of Quantification (LOQ),” Organic & Medicinal Chemistry International Journal, vol. 7, no. 5, Aug. 2018, doi: 10.19080/OMCIJ.2018.07.555722.

[44] B. Cassidy, T. Bloomingdale, and J. Carmody, “Navigating ICH Q2(R2) compliance in analytical method validation: A gap analysis toolkit to streamline risk assessment and change management,” J. Pharm. Sci., vol. 114, no. 6, Jun. 2025, doi: 10.1016/J.XPHS.2025.103749.

[45] S. S. Shinde and Dr. P. Khulbe, “Analytical Method Validation: A Comprehensive Review of Current Practices,” South East. Eur. J. Public Health, pp. 244–254, May 2025, doi: 10.70135/SEEJPH.VI.6516.

[46] V. Camellia et al., “The Effect of Chronic Administration of Amphetamine-Type Stimulants on Oxidative Stress and Inflammation in Wistar Rats,” Baghdad Science Journal, vol. 21, no. 7, pp. 2237–2245, 2024, doi: 10.21123/BSJ.2023.9170.

[47] R. S. Alharbi, A. H. Alhowail, A. G. Alharbi, and A. M. Emara, “Evaluation of the health status outcome among inpatients treated for Amphetamine Addiction,” Saudi J. Biol. Sci., vol. 29, no. 3, pp. 1465–1476, Mar. 2022, doi: 10.1016/J.SJBS.2021.11.025.