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Development and Validation CFIA / MZ System as a Green Method for Determination of Thiol Drug (D-PEN)

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Abstract

A simple, automated, and sensitive continuous flow-injection analysis/merging zones technique (CFIA/MZ) method was developed for the determination of D-Penicillamine (thiol drug) in pure, pharmaceutical formulations and biological samples. This method involved the reaction of 1,2-Naphthoquinone-4-Sulphonic acid Sodium Salt (NQS) with D-Penicillamine to produce a brown-colored complex that has maximum absorbance at 463 nm. The method was cheap, economic, precise and accurate where the limit of detection was 6.3 µg.mL^{-1} and the RSD% was 0.34 and Recovery was 99.31%. Various chemical and physical conditions that affected the reaction have been studied. A wide calibration curve was rectilinear within the concentration range (15-1000) µg.mL^{-1} with a sample throughput of 103 samples.hour^{-1}. The proposed procedure was applied successfully for the estimation of D-PEN in pharmaceutical and biological samples and the results obtained were favorably compared with those given by a reference method of United States Pharmacopoeia (USP), and there was no significant difference between the obtained results, regarding accuracy and precision at the 95% confidence level.

Keywords: D-Penicillamine, CFIA/Spectrophotometric system, A green chemistry, Biological samples, D-PEN, CFIA/MZ, Spectrophotometric estimation.

1. Introduction

D-Penicillamine (D-PEN) ($C_5H_{11}NO_2S$), M.wt = 149.212 g.mol^{-1}, the IUPAC name is (2S)-amino-3-methyl-3-sulfanylbutanoic acid [1], Figure (1) shows the chemical structure of D-PEN.

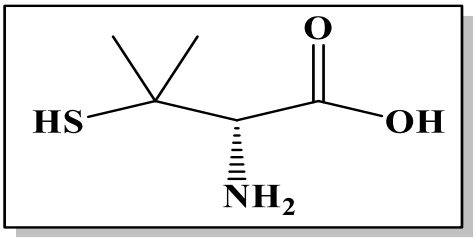

Figure (1): formula structure of D-PEN.

D-PEN is sold under the trade names of Cuprimine among others, is a medication primarily used for the treatment of Wilson's disease [2].

It is also used for people with kidney stones who have cysteine, rheumatoid arthritis, and various heavy metal poisonings[2,3]. It is taken by mouth [3]. A thiol drug is official in the United States Pharmacopoeia (USP) [4], which describes HPLC methods for the determination of D-PEN either in raw material or in pharmaceutical formulations.

Several methods have been reported for the determination of D-PEN in pharmaceutical dosage forms including kinetic spectrophotometry [5], electrochemical chiral sensor [6], Flow-injection [7], Circular dichroism spectroscopy [8], spectrofluorimetric [9], Fluorometric sensor [10], HPLC [11,12], carbon paste electrode [13], HPLC-FLD and CE-LIF [14], spectrophotometric [15,16], potentiometric [17], fluorescence [18-20], Electrochemical sensor [21], Electrocatalytic [22], Chemiluminescence [23] and Voltametric [24].

Although the procedures are specific, most of the described methods are time consuming and require multistage extraction procedures. On the other hand, the reported spectrophotometric methods suffer from one or the other disadvantage such as poor sensitivity, use of organic solvent, the problems of extraction scrupulous control of experimental variables and special equipment, or small ranges. The proposed method of Flow Injection Analysis / Merging Zones (CFIA/MZ) technique is simple, accurate with high repeatability of the results obtained, do not need for further treatment for the samples as well as is not costly and do not require an expensive or toxic reagent,

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besides the short time required for the analysis with highly sampling per hour [25-28].

Apparatus and FIA Manifold

All of spectral absorbance quantifications were applied on an Optima VIS 9200, digital single beam that record spectrophotometer with flow cell (quartz silica, 1cm) with 80µL internal volume is inside the detection unit and 1cm an optical path length using for the absorbance measurements as average peak height expressed in mV (n=3).

A one channel manifold Flow Injection Analysis-merging zones for spectrophotometer estimation of D-PEN was suggested in this article. A power supply (Yaxun, 1501AD, China) with Peristaltic pump (Master flexC/L, two channel USA) using for pump a carrier stream (distilled water), and solutions were passed through the injection valve (homemade); five-three-way injection valve that if to valve must be contains three loops were made of Teflon. chemicals and reagents solutions which based on merging zones version [25,26]. The injection valve that used to supplied suitable volumes that were injected of standard solutions and samples. The tubes were made of flexible vinyl with 0.22mm (I.D) using for the peristaltic pump; mixing and reaction coil that was manufactured from glass with 2mm (I.D). All of the parts of the CIFA as shown in Figure (2) with details. A carrier stream was distilled water that was joined with injected sample D-PEN in L1 and merged with sodium phosphate buffer (pH=12) NaH_2PO_4 and 1,2-Naphthoquinone-4-Sulphonic Acid Sodium Salt (NQS) as reagent in L2 and L3 respectively. Then mixed all together in a mixing coil that has a length of 70 cm and flow rate of carrier 12.8 mL.min^{-1}. The maximum absorption was found at 463 nm brown-coloured for D-PEN and NQS complex as peak height in (mV).

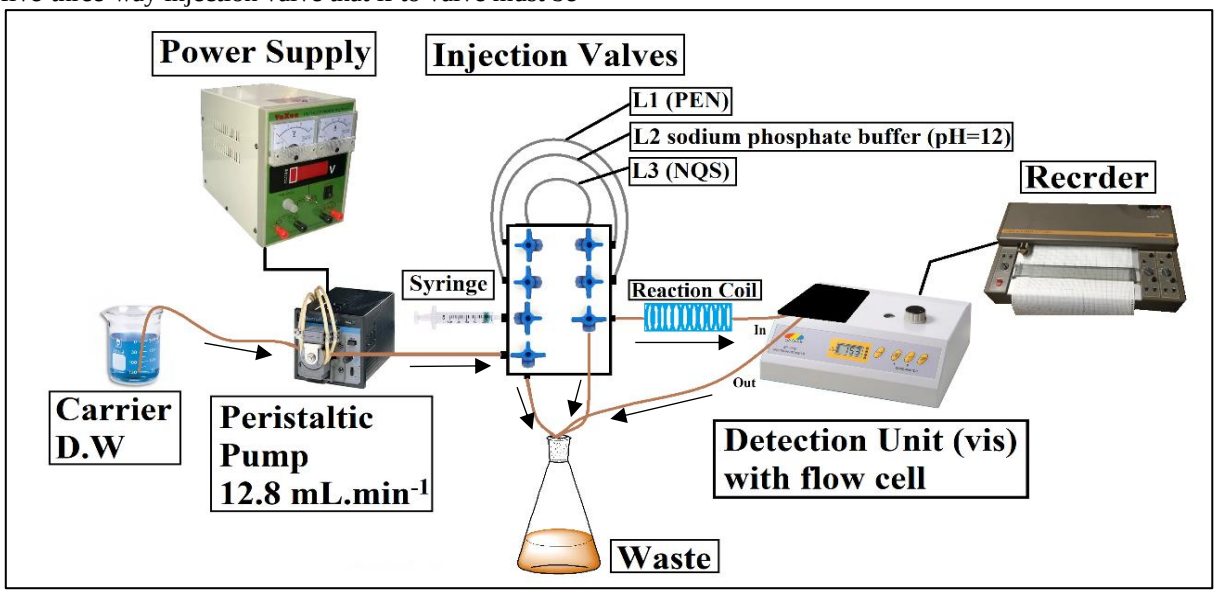


Figure (2): The developed CFIA system.

Chemicals and reagents

All the chemical materials and reagents employed were of analytical class and all the solutions preparing always used.

D-PEN stock solution (1500 µg.mL^{-1} = 1×10^{-2} M): A 0.15 g amount of pure D-PEN (M.wt = 149.21 g.mol^{-1}, Merck, Germany) was dissolving in distilled water then be consummated to 100 mL in the standard volumetric flask with distilled water. More the diluted solutions preparing by adequate diluting of the stock standard solution with distilled water.

NQS solution (2.70×10^{-2} M): A 0.7012 g amount of NQS (M.wt = 260.20 g.mol^{-1}, Merck) was dissolved in the mount of water then the volume was made to 100 mL in a standard volumetric flask and farther dilution to these solutions to obtain desired concentrations The solution was freshly prepared and protected from light during use.

Buffer solution (pH = 12) was prepared by mixing 100 mL of 0.2 M aqueous solution of sodium dihydrogen phosphate NaH_2PO_4 with 200 mL of 0.2 M sodium hydroxide NaOH in 100 mL volumetric flask, and adjusted by pH meter.

Batch method

An increasing volumes (0.1-2) mL of D-PEN was transferred into a set of 10mL standard flask; after that 0.5 mL of NQS (2.70×10^{-2} M) were added, then 1.5 mL of the buffer solution was added and consummate the volume of the solutions to the mark with distilled

water. The maximum absorption of the brown complex was found at λ_{max} 463 nm versus to the blank solution.

Pharmaceutical preparations of D-PEN (1000 µg. mL^{-1})

Pharmaceutical formulations were gained from trading sources obtainable capsules by choosing 20 capsules from three kinds of companies were assayed by the proposed procedure. The various providers from different companies were containing:

1. Artamin (250 mg) VHB Life Sciences Limited, India.
2. D-Pencellamin (250 mg) SAMARTH LIFE SCIENCES PVT LTD, India.
3. Cuprphen (250 mg) Laboratorios Rubió SA, Spain

Every 20 capsules from each source were weighed exactly and mixed, then a weigh of average one capsule was taken which contain 250 mg of D-PEN. Each weight that taken was treated as pure material in the procedure mentioned above. Further solutions were diluted to preparing the concentration inside of the linearity of the calibration graph. A recovery experiment was performed by applying the standard-addition technique [29]. The recovery was assessed by determining the agreement between the measured concentration and the final known concentration to the sample. Each test was repeated three times.

The limits of detection (LOD) and quantification (LOQ) were calculated according to [28]:

$$\text{LOD} = \frac{3.3\ SD}{b} \text{ and LOQ} = \frac{10\ SD}{b}$$

Urine samples preparation

The sample was collected from a healthy volunteer and stored at 20 °C until use after gentle thawing. For urine sample preparation a volume of 1 mL converted to a volumetric flask 10 ml and spiked with (2, 4) mL of standard solution (500 µg.mL^{-1}) and diluted with distilled water to attain (100, 200) µg.mL^{-1} of D-PEN [28].

Result and discussion

Absorption spectra

A final concentration of 20 µg.mL^{-1} D-PEN (1.3×10^{-4} M) was reacted with (1.3×10^{-3} M) of NQS in a basic medium to give the colored product complex which was examined under visible spectrum (from 350-650) in order to determine the maximum absorbance for the complex and it was clear that the λ_{max} was 463nm for the D-PEN-NQS complex as shown in Figure (3).

Study of stoichiometry complex of NQS with D-PEN drug

In order to know the ratio of reaction that occurs between the reagent and the drug two important way were proceed which is mole ratio method and continuous variation method (Job's method) and the result shown that D-PEN produced a 1:1 complex with NQS as shown in Figure (4):

1. Mole ratio: (0.01 M of each D-PEN and NQS mix in order to the procedure of mole ratio and complete to 10 mL with distilled water in the present of 0.2 M sodium phosphate as buffer solution (pH = 12).
2. Job's method: (0.01 M of each D-PEN and NQS mix in order of Job's method and complete to 10 mL with distilled water in the present of 0.2 M sodium phosphate as buffer solution (pH = 12).

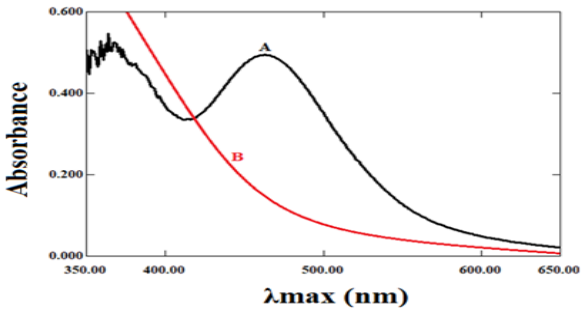


Figure (3): The Absorption spectrum of A/ D-PEN-NQS complex against blank solution (NQS and sodium phosphate buffer), B/ blank solution against distilled water.

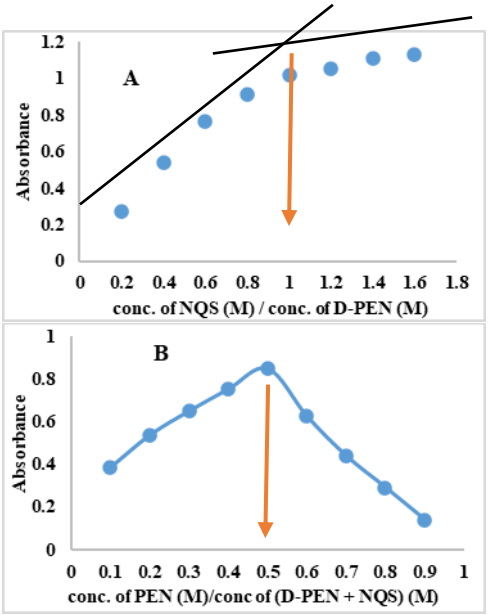


Figure (4): The complexation ratio between a reagent with drug, A/ mole ratio for D-PEN-NQS complex, B/ job's method for D-PEN-NQS complex.

The proposed mechanism of the reaction

Depending on the results collected from the mole ratio and Job's methods it is clear that the D-PEN–NQS complex associate in 1:1 ratio so the proposed mechanism below is likely to suggest as shown in Scheme (1). NQS could react with amino group of primary amine derivative. So, amino group of D-PEN also, taking on nucleophilicity due lone electron pair of nitrogen atom, trend to attack on the electron–deficient centre in NQS, namely no.4 carbon

atom (3,4-C=C carbon bond conjugate with 2-C=O, as a result 4-C of NQS becomes electron lacking centre). So, it is concluded that amino group of D-PEN react with 4-sodium sulphonate of the reagent molecule, to form brown N-alkyl-amino-naphthoquinone [16].

Preliminary investigation

Effect of NQS

The effect of NQS volume was examined with 20 µg.mL^{-1} D-PEN. It has been monitored that the volume that gives the highest absorbance was 0.5 mL of 2.7×10^{-2} M NQS and this volume was selected for subsequent experiences, as shown in Figure (5-A).

Effect of sodium phosphate buffer

The influence of medium was studied carefully due to it was directly affected on the coupling of D-PEN with NQS. A series volume (0.25-1.25) mL of sodium phosphate buffer (pH=12) was used for the experiment in the presence of a final concentration of NQS (1.3×10^{-3} M), and a suitable Final concentration of 20µg.ml^{-1} D-PEN. The absorbance increases with observed up to 1.5 mL than decrease, so this volume was chosen the optimum buffer solution as a suitable medium for the formation of colored product, as shown in Figure (5-B).

Calibration curve of the classical method

Transfer a series of volumetric flask (10 mL) containing an increasing volume (0.1 – 2 mL) standard solutions of D-PEN (200 µg.mL^{-1}) Then 0.5 mL of NQS (2.7×10^{-2} M), then added 1.5 mL of buffer (pH = 12). The solutions had been diluted to the mark with distilled water. The standard solutions were measured at 463 nm against blank solution prepared in the same way without D-PEN. The standard curve was constructed and linear range (2-40) µg.mL^{-1} for the estimation of D-PEN, as shown in Figure (6).

Accuracy and precision

Under the ideal conditions described in the established method, accuracy and precision were studied through measuring two different concentrations of D-PEN, and according to the results that have been reached as shown in Table (1) show that the classical method has good with high accuracy and precision; each measurement is repeated for five times.

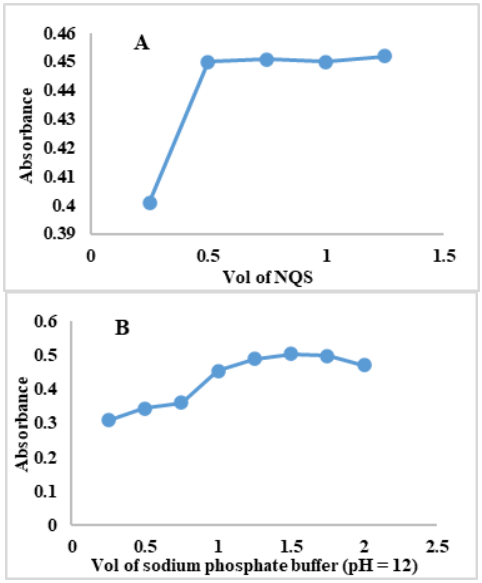
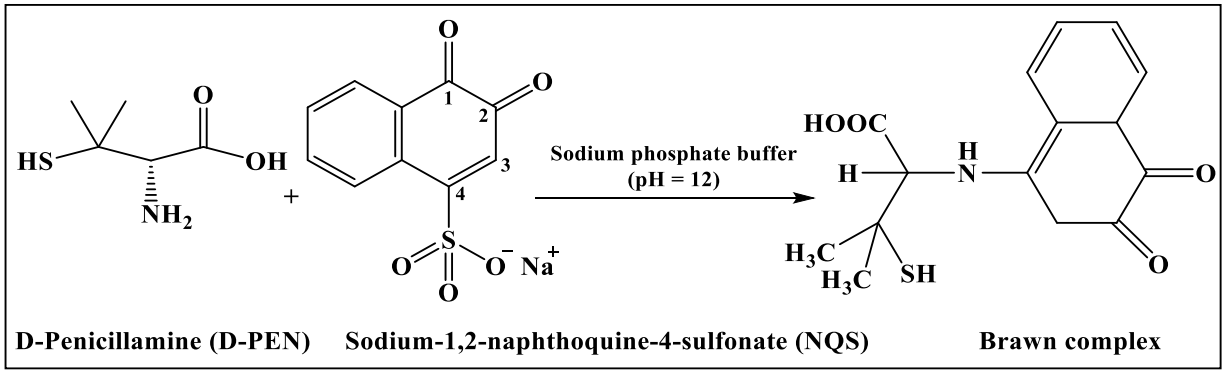


Figure (5): Chemical parameter for batch, A/ volume of NQS, B/ volume of sodium phosphate buffer.

Calculations of stability constant

Calculated static stability for the proposed interaction (D-PEN: NQS) was calculated depending on the two groups of solutions were prepared; the first group of solutions were placed to include a stoichiometric lot of D-PEN to reagent NQS, while the second group was placed to include two-fold excess of NQS. According to the proposed mechanism and stoichiometry ratio between the reagent and drug (1:1**)**. The reaction between D-PEN and NQS proceeds according to the equation [30]:



Scheme (1): The proposed mechanism of the complex between D-PEN with NQS.

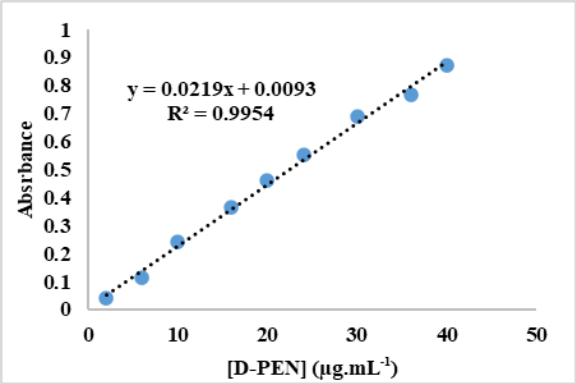


Figure (6): Linear calibration carve of D-PEN –NQS using spectrophotometric method.

$$D + R \rightleftharpoons DR$$

α α (1 – α)

$$K = \frac{[DR]}{[D][R]}$$

$$K = \frac{(1-\alpha)}{\alpha^2 C}$$

While (K) is the stability constant, (C) is the molar concentration (M) of the product which is equivalent to the concentration of D-PEN (١×10$^{-٣}$ M), (α) is the degree of dissociation can be written as follows:

$$\alpha = \frac{Am - As}{Am}$$

Where Am; As are the values of absorbance of the aqueous solution including a more than enough and stoichiometric amount of the reagent. The spontaneous of complex formation reaction (ΔG value) was estimated based on K evaluation as in Table (2) and the equation: **ΔG = -RT lnK**

ΔG: Gibbs free energy,
R: general constant of gases (8.314 J. mol^{-1}. K^{-1}),
T: absolute temperature (298.15 K)

Flow injection/ Merging zones spectrophotometric determination

After selecting the optimum conditions of reaction of D-PEN with NQS in presence of sodium phosphate buffer in the classical spectrophotometric method. The spectrophotometric reaction was automated with flow injection-merging zones technique to study the best practical parameters and to obtain spectral automated with a fast way to estimate D-PEN. So, the batch procedure for estimation of D-PEN was employed as a basis to develop flow injection analysis method.

Manifold of the flow injection system

After installing the system and linked portions, been the study of optimal design of homemade FIA system. The developed system is shown in Figure (2) is composed of one line supplies the carrier is distilled water leading to the injection valve; which contain three loops (different loop length with 0.5mm I.D.) that fill by the drug, reagents and buffer according to the order D-PEN in L1, NQS in L2 and, sodium phosphate buffer (pH=12) in L3.

Optimization of the developed FIA system conditions

Initial studies were directed towards the optimization of the experimental conditions for MZ-FIA system.

Chemicals and physical variables

Effect of chemical variables (concentration of reagent, the type of buffer, the value of pH, and the order of addition) and physical parameters (the flow rate, length of reaction coil sampling, injection time and the injected volume of drug, reagent and buffer) were studied.

Effect of NQS

The optimum concentration of the reagent NQS was studied by injecting different concentrations (3.3×10^{-4}-5.4×10^{-3}) M using a homemade injection valve loading in (L2). The results in Figure (7) indicated that the 1.3×10^{-3} M gave the highest value of absorbance expressed as peak height in mV (n=3) with high repeatability.

Table (1): Accuracy and precision.

D-PEN (µg. mL^{-1})		E (Error = $\bar{x}$ - µ)	Rec.	Er.	RSD%
Present µ	*Found $\bar{x}$				
20	20.443	0.854	102.215	2.215	1.770
40	38.297	-1.703	95.74	-4.258	2.455

Average of five determinations.

Table (2): Stability constants and Gibbs free energy of the reaction.

	Am	As	α	K (L.mol^{-1})	ΔG (J.mol^{-1})
1	١,٠٥٨	١,٠٢٣	0.033	8.8×10^5	-33939
2	1.055	1.014	0.039	6.4×10^5	-33126
Average				7.6×10^5	-33533

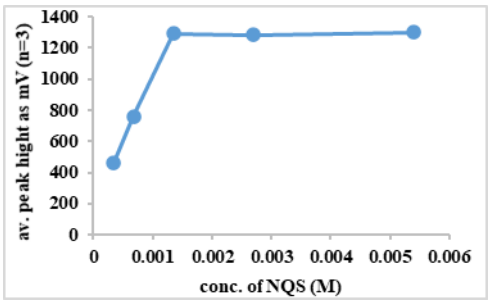


Figure (7): Effect of NQS (D-PEN = 100 µg.mL^{-1}).

Effect of the type of buffer

Three buffer solutions Acetate buffer (pH = 3), CH_3COOH\ CH_3COONa\ NaCl, Phosphate buffer (pH = 7) K_2HPO_4\ KH_2PO_4 and sodium phosphate buffer (pH = 12) NaH_2PO_4\ NaOH were studied by injecting the buffer solution in L3. The results in Figure (8-A) show that sodium phosphate buffer gave the highest value of absorbance expressed as peak height in mV (n=3) with high repeatability. The optimum value of pH was studied by preparing six solutions of sodium phosphate buffer with different values of pH (2-12) using a homemade injection valve loading in (L3). The results in Figure (8-B) indicated that the buffer solution pH=12 of sodium phosphate gave the highest value of absorbance (mV) and was chosen for subsequent experiments.

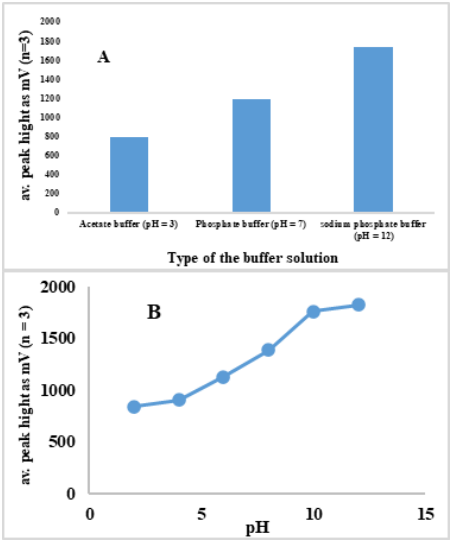


Figure (8): Effect of, A/ The type of buffer, B/ pH.

Choosing the best manifold unit

The results in Figure (9) indicated that the best sequence is (D in L1 + B in L2 + R in L3) where D is D-PEN, R is NQS, B is the Buffer solution (pH = 12).

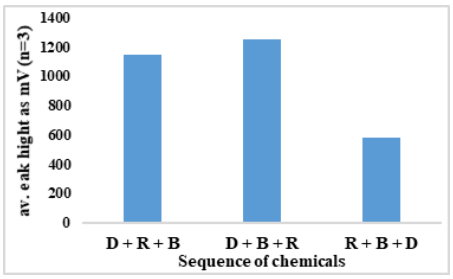


Figure (9): Effect of the sequence of chemicals.

Effect of injected volume and reaction coil

For D-PEN–NQS reaction, the best loop volumes for drug, buffer, and reagent were (78.50-117.75-58.88) µL and the best reaction coil length was 70 cm as shown in Figure (10). The figure shows the important parameters for the best loop volumes that used for the determination of D-PEN for the NQS system.

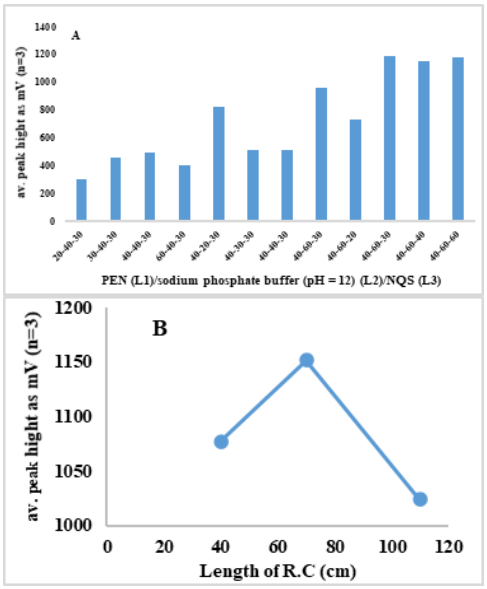


Figure (10): Effect of A/ Injected volume B/ Reaction coil.

Effect of optimum total flow rate and sample through-put

All available flow rates were studied for the system and that shows the best flow rate for (D-PEN-NQS) FIA system was 12.77 mL.min^{-1} with sample through-put about 103 sample.hour^{-1} as shown in Figure (11) the sampling rate was calculated depending on the time required for loading the drug, and the reagent to loops of the seven three-way valve plus the time required to maximum peak height appear (this time was found to be 22 s) in addition to 13 s required for loading drug, buffer and reagent, so the sampling rate was 103 sample.hour^{-1} for the developed FIA system.

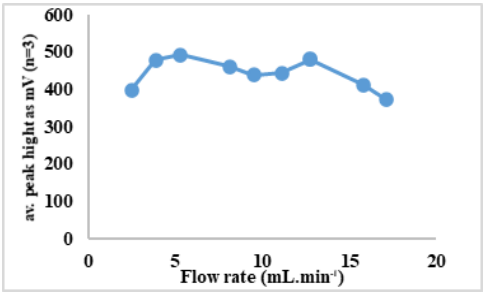


Figure (11): Effect of total flow rate.

Purge time

Purge time for the sample segment to be injected via the carrier stream (distilled water) was studied, using the ideal chemical and physical parameters were studied previously. for D-PEN-NQS system time like (5,10,15 and 20) sec. and open valve (injected mode) were used, and showed that the purge time more than 20 sec. giving the highest response intensity. For this reason, the open valve was selected as an optimum purge time to complete transportation of sample from sample loop to flow cell, the results obtained were summarized in Figure (12).

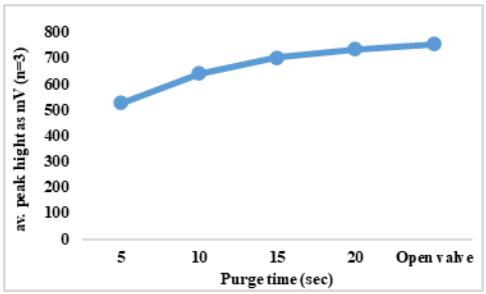


Figure (12): Effect of purge time on peak height in mV for D-PEN-NQS (100 µg.mL^{-1})

Dispersion of zone

Dispersion is a physical phenomenon that occurs in flow injection technique as a produce of the influence of different concentration solutions, the sample mixed with carrier stream and then spread the sample in the solution. Success of the analysis process by FIA on three principles [31]:

1. Reproducible injection time.
2. Reproducible sample injection volume.
3. Control on the dispersion of the sample zone.

The dispersion of the D-PEN-NQS reaction was 1.223 as shown in Figure (13) and Table (3) The dispersion was calculated according to the law:

$$D = \frac{C_o}{C}$$

While, C_o is the peak height without dilution (conducting interaction outside the flow injection system), C is peak height with dilution (conducting interaction inside the flow injection system). The study was conducted with two experiments, in the first experiment, mixed all the ingredients interact in a suitable beaker and then pass the solution through the flow injection system (as carrier stream) to get fixed response represented (C_o). In the second experiment, D-PEN into L1, sodium phosphate buffer in L2 and NQS in L3. Distilled water pass through the system as a carrier (12.8 mL.min^{-1}) and the component injected, works to push the components to reaction coil and then to the detector to get response represented by (C).

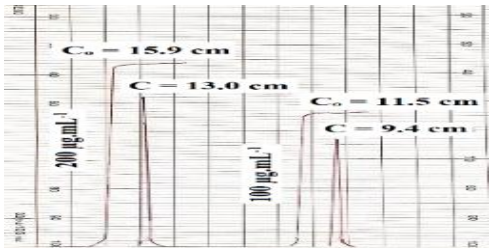


Figure (13): Dispersion of D-PEN in CFIA system.

Table (3): Dispersion value of D-PEN.

D-PEN Conc. µg.ml^{-1}	C_o (cm)	C (cm)	D
100	11.5	9.4	1.223
200	15.9	13.0	1.223

Calibration curve

After verbal and verification of all optimum condition, a series of D-PEN concentration (from 10 µg.mL^{-1} to 1500 µg.mL^{-1}) were prepared and injected to FIA system with NQS and buffer solution in order to know the optimum range of D-PEN concentration which can be applicable for this method and it shows that the best concentration range extends (15-1000) µg.mL^{-1} as shown in Figure (14) and Table (4).

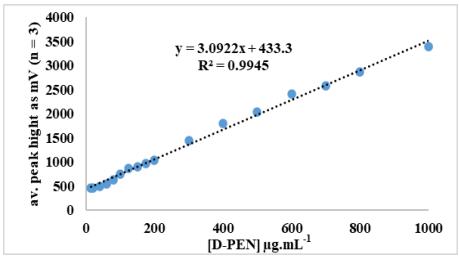


Figure (14): Linear dynamic range for spectrophotometric determination of D-PEN using the developed CFIA system.

Analysis of variation (ANOVA)

Calculate the sum of squares of the difference of values y_i (response) from $\hat{y}_i$ (appraiser response), (imply error) and called (about regression) to obtain $\Sigma (y_i - \hat{y}_i)^2$ for (n_2) freedom degrees to get the sum of squares $(S_o)^2$ [32,33].

Calculate the sum of squares of the variance of values y_i from average value $\bar{y}$ (due to regression) to obtain $\Sigma (\hat{y}_i - \bar{y})^2$ and for (1) of degrees of freedom to obtain the sum of squares $(S_1)^2$, when dividing the $(S_1)^2$ on $(S_0)^2$ get the value (F) as shown in the Table (5).

$F_{crit.}$ (4.1491) << F (36.5259) so it may be complete which there is an important relationship between the concentration of D-PEN and the signal got.

Methods validation

The analytical characteristics just as correlation coefficient (r), detection limit, linear range and relative standard deviation of each procedure were estimated [30] at the improved conditions; as shown in Table (6). A calibration curve was constructed Figure (14) for a set of D-PEN standard solutions and the basic analytical figure of deserts of the proposed method. Statistical assessment of regression line presented the results of standard deviation for residuals ($S_{\underset{x}{y}}$); intercept (Sa) and slope (Sb) under 95% confidence limits for $(n-2)$ freedom degrees were clarified in the table. The small subjects were shown the high repeatability of the results obtained with high reproducibility of the proposed CFIA technique compared with the batch method.

Flow injection analysis/merging zones was easier and simple because that was rapid in analysis (sample throughput of 103 sample.hour^{-1}); a large linear scale of calibration curves was gotten.

Table (4): Calibration table as S.E.M for D-PEN-NQS

conc. of D-PEN (µg.mL^{-1})	peak height (mV)			Average response ($\bar{y}$) (mV)	SD	RSD%	S.E.M	*E/y%
15	456	456	448	453	4.62	1.02	453±11.47	2.53
20	464	464	472	467	4.62	0.99	467±11.47	2.46
40	480	480	488	483	4.62	0.96	483±45.87	2.38
60	544	536	536	539	4.62	0.86	539±11.47	2.13
80	632	632	624	629	4.62	0.73	629±11.47	1.82
100	752	752	760	755	4.62	0.61	755±11.47	1.52
125	880	872	848	867	16.65	1.92	867±41.34	4.77
150	896	896	912	901	9.24	1.02	901±22.93	2.54
175	952	968	960	960	8.00	0.83	960±19.86	2.07
200	1040	1048	1040	1043	4.62	0.44	1043±11.47	1.10
300	1440	1448	1440	1443	4.62	0.32	1443±11.47	0.79
400	1800	1800	1820	1807	11.55	0.64	1807±28.67	1.59
500	2040	2060	2020	2040	20.00	0.98	2040±49.65	2.43
600	2400	2440	2380	2407	30.55	1.27	2407±75.84	3.15
700	2600	2600	2560	2587	23.09	0.89	2587±57.33	2.216
800	2860	2900	2820	2860	40.00	1.40	2860±99.30	3.472
1000	3380	3400	3420	3400	20.00	0.59	3400±49.65	1.46

* $\frac{E}{y}\% = t_{tab}\frac{SD}{\sqrt{n}} \times \frac{100\%}{\bar{y}}$

Table (5): Analysis of variation for developed FIA method

Source of Variation	sum. of squares (SS)	df	mean of squares (MS)	F ($\frac{S_1^2}{S_2^2}$)	F crit
Between Groups (Error)	16445838.91	1	16445838.91 = S^2_2	36.5259	4.1491
Within Groups (Regression)	14408042.18	32	450251.32= S^2_1		
Total	30853881.09	33			

Table (6): Analytical characteristic of calibration carve for [D-PEN-NQS-sodium phosphate buffer (pH=12)] CFIA system.

Parameters	FIA method	Batch method
λmax (nm)	463	
Regression equation; y = bx+ a; y = absorbance; x = concentration (µg. mL^{-1})	y = 3.0922x + 433.2978	y = 0.0219x + 0.0093
Linear range (µg. mL^{-1})	15-1000	2-40
Average of recovery (Rec%)	99.31	98.98
Average of Relative Error % (Erel%)	-0.6856	-1.0217
Average of Relative standard deviation (RSD%)	0.3441	3.0403
Slope (b); (mL. µg^{-1}) $b = \frac{\sum_i[(xi-\bar{x})(yi-\bar{y})}{\sum_i(xi-\bar{x})^2}$	3.0922	0.0219
Intercept (a); $a = y - bx$	433.2978	0.0093
Determination coefficient (R^2)	0.9945	0.9954
Correlation coefficient (r): $r = \sum_i[(xi-\bar{x})(yi-\bar{y})]\sqrt{(\sum_i(xi-\bar{x})^2)\,(\sum_i(yi-\bar{y})^2}$	0.9972	0.9977
Standard deviation of slope (Sb) $Sb = \frac{S_{\underset{x}{y}}}{\sqrt{\sum_i(xi-\bar{x})^2}}$	0.0593	0.0006
Standard deviation of intercept (Sa) $Sa = S_{\underset{x}{y}}\sqrt{n\sum_i(xi-\bar{x})^2}$	25.4091	0.0135
Limit of detection (LOD)	6.3372	1.2516
Limit of quantification (LOQ)	21.1240	4.1719
Molar absorptivity (Ɛ) (L/mol.cm) $\varepsilon = b \times M.Wt \times 1000$	-------	3267.74
Sandell s sensitivity (S) (µg.cm-2) $S = \frac{M.Wt}{\varepsilon}$	-------	0.0457
Sample through put (h^{-1})	103	5
Standard deviation of the residuals; $S_{\underset{x}{y}} = \sqrt{\frac{\sum_i(yi-\hat{y}_i)^2}{n-2}}$ $\hat{y}_i = bxi + a$	72.3962	0.0212
Confidence limit of slope (b) $CL_b = b \pm t \times Sb$	3.0922 ± 0.1257	-------
Confidence limit of intercept (a) $CL_a = a \pm t \times Sa$	433.2978 ± 53.8672	-------

Study of interferences

In order to examine the selectivity of the suggested method, the interference likely to be introduced from excipients and for capsules weight adjustments (as sucrose, cellulose, lactose, glucose and sodium citrate) was studied. A sample of pure 20 µg.mL^{-1} D-PEN spiked with half, equal and double fold excess concentration of selected interferences and 1.3×10^{-3} M NQS in presence of 0.2M of sodium phosphate buffer, three excipients were analyzed. The acceptable recovery values (95-105%) demonstrated that, there were no interferences during the determination of D-PEN using new CFIA system, the results summarized in Table (7).

Applications and assessment of the suggested method

Pharmaceutics

According to the standard addition process [34,35], three types of capsules containing D-PEN have been analysed under the proposed method which equipped with different origins.

The statistical comparison between the proposed method with official method USP-HPLC procedure as our reference method [4] using the student t-test and F-test showed that the calculated F-test values were (7.3690) and (6.6573), t-test values were (0.2721) and (0.4640) less than the theoretical F-test (19.00) and t-test (2.78) via CFIA/merging zones and batch methods, respectively.

Urine samples

The FIA method was further applied effectively for the estimation of D-PEN in spiked human urine samples.

100 µg.mL^{-1} of D-PEN was tested for accuracy and precision. Each concentration was analysed thrice. Acceptable accuracy and precision for urine samples were observed in the Table (9).

Table (7): Interferences effect on [D-PEN –NQS-sodium phosphate buffer] FIA system.

Type of Interference	conc. of Interferences (µg.mL^{-1})	Average response ($\bar{y}$) (mV)	Erel%	Rec%
Standard	20	495	-0.2328	99.77
	10	496	1.3841	101.38
Sucrose	20	497	3.0011	103.00
	40	498	4.6181	104.62
	10	495	-0.2328	99.77
Cellulose	20	493	-3.4668	96.53
	40	497	3.0011	103.00
	10	496	1.3841	101.38
Lactose	20	498	4.6181	104.62
	40	495	-0.2328	99.77
	10	493	-3.4668	96.53
Glucose	20	494	-1.8498	98.15
	40	494	-1.8498	98.15
	10	495	-0.2328	99.77
Sodium citrate	20	496	1.3841	101.38
	40	497	3.0011	103.00

Table (8): Applications of the proposed methods compared with official method (USP) for estimation of D-PEN in medicinal formulations.

Dosage form	Proposed FIA method						Official method (theoretical)					
	conc. of D-PEN (µg.mL^{-1})		Erel %	Rec %	Mean Rec %	RSD%	conc. of D-PEN (µg.mL^{-1})		Erel %	Rec %	Mean Rec %	RSD%
	Present	Found					Present	Found				
Artamin (250mg) VHB Life Sciences Limited, India.	100	102.34	2.34	102.34	100.89	1.89	100	100.9٠	0.9٠	100.9٠	100.46	0.7١
	200	198.89	-0.5٦	99.4٥		1.48	200	200.05	0.02	100.0٣		0.5٧
D-pencellamin (250mg) SAMARTH LIFE SCIENCES PVT LTD, India.	100	100.53	0.53	100.53	100.8٥	1.92	100	99.98	-0.02	99.98	100.25	0.71
	200	202.33	1.16	101.1٧		1.45	200	201.05	0.52	100.5٣		0.5٧
Cuprphen (250mg) Laboratorios Rubió SA, Spain	100	98.48	-1.52	98.48	100.42	1.96	100	99.5٠	-0.5٠	99.5٠	100.33	0.7٢
	200	204.73	2.36	102.3٧		1.43	200	202.32	1.16	101.16		0.56

$$t_{tab} = 2.78 \; for \; n_1 = n_2 = 3, n_1 + n_2 - 2 = 4, at \; 95\% \; confidence$$
$$F_{tab} = 19.00 \; for \; n_1 - 1 = n_2 - 1 = 2, at \; 95\% \; confidence$$

Table (9): Determination of D-PEN in urine samples using suggest CFIA system.

Sample	Added Conc (µ). µg.mL^{-1}	Found Conc($\bar{x}$). µg.mL^{-1}	Erel %	Rec. (%)	RSD (%)
1	100	101.13	1.1254	101.13	0.1268
2	100	100.80	0.8020	100.80	0.5558
3	100	102.42	2.4190	102.42	0.4938

Table (10): Different analytical methods for determination of of D-PEN.

Analytical method	Comment	LOD	Linear range	Ref.
kinetic spectrophotometry	It is based on the redox reaction where the thiol compound (RSH) reduces CuII-neocuproine complex to CuI - neocuproine complex.	2.4×10^{-7} mol.L^{-1}	(8.0×10^{-7}-8.0×10^{-5}) mol.L^{-1}	5
electrochemical chiral sensor	The preparation of the modified electrode was a two-step procedure in which electrodeposition of ZnO was conducted first on the ITO glass substrate.	23.56 mM	(5-30) mM	6
Circular dichroism spectroscopy	Basically, DPA and LPA provide very low CD signals. However, the CD signals of DPA and LPA can be enhanced in the presence of Cys-CdS QDs. The CD spectra of DPA and LPA exhibited a mirror image profile	0.49 µM	(1-35) µM	8
Fluorimetric sensor	In this optical sensor, carbon dots (CDs), which were synthesized from saffron, were used as a fluorophore.	0.02 µg.mL^{-1}	(0.05-13.0) µg.mL^{-1}	10
HPLC	Chromatographic analysis was performed by Waters RP-HPLC system using a Symmetry® C (18) column with a mobile phase comprising 0.1 % formic acid and methanol (95:5 v/v).	…….	(8-96) µg.mL^{-1}	11
carbon paste electrode	The morphologies of Ag-ZnO nanoplates were examined by scanning electron microscopy. It was found that under an optimum condition (pH 7.0).	0.015 µM	(0.03-250.0) µM	13
(CFIA/MZ) proposed method	This method involved the reaction of 1,2-Naphthoquinone-4-Sulphonic acid Sodium Salt (NQS) with D-Penicillamine to produce a brown-colored complex that has maximum absorbance at 463 nm. The sample throughput was 103 samples.hour^{-1}	6.3 µg.mL^{-1}	(15-1000) µg.mL^{-1}	----

Conclusions

By reviewing the literature in the field of injection analysis, few researchers found that they used this technique depended on merging zone of chemicals to estimate D-PEN that is why a research plan for this manuscript was proposed for the sensitive spectrophotometric estimation of thiol drug in pure form, dosage samples and urine via a new CFIA design. It is characterized by a wider calibration range, high sampling rate. These methods can be employed for the regard of µg.mL^{-1} amount of D-PEN without indigence for the prior divorce action, temperature or pretreatment of specimen and solid phase extraction. The capital benefit of the methods is its huge workings range; suitable sensitivity and its proper for appropriate in routine examination in pharmaceutics specify control laboratories due to their expertness and their result in decrease reagents waste and toxicity of organic reagents when comparison with batch methods and official (USP) HPLC method.

Search objective

Study and determination of D-PEN in pure and pharmaceutics using modified spectrophotometric method via continuous FI system / merging zone technique. Apply the proposed method to pharmaceutical drugs. Analysis of obtained data statistically using analysis of variation (ANOVA), t-test and F- test. Comparison of the developed method with standard traditional methods for determination of D-PEN.

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