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A novel approach for the spectrophotometric determination of tryptophan in drug samples of different origins using homemade FIA / merging zones techniques

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ABSTRACT

Anew, rapid and sensitive batch and flow injection – merging zones spectrophotometric methods for the determination of Tryptophan in pure material and pharmaceutical preparations were proposed. These methods based on oxidation of the reagent Diphenyl amine sulphonate (DASA) to diphenyl benzidine sulphonic acid after reaction with sodium nitrite in the sulphuric acid medium. The unstable oxidation product react quickly with sodium nitrite to produce a diazotized intermediate. The diazotized intermediate is coupled with Trp., a pink color product was developed which is stable for 1 h in 30^{0}C. The colored product measured at 522nm. Optimum concentration of chemical reactants and physical instrumental conditions have been investigated. A linear graph of peak high versus concentration indicate that Beer's low is obeyed over the concentration range of 3-40 and 5.100µg. ml^{-1} of Tryptophan with detection limits of 0.2325 and 0.188 µg.ml^{-1} of Tryptophan for batch and FIA methods, respectively. The optimized FIA system was able to determine Trp. with a through put 52 samples.h^{-1}. The proposed methods were applied successfully for the determination of Tryptophan in pharmaceutical preparations and statistical analysis of the results were compared with results by the British pharmacopeia were also reported. © 2016 Trade Science Inc. - INDIA

KEYWORDS

Diphenyl amine sulphonate;
Tryptophan;
Contineous – flow injection;
Azo – coupling reaction

INTRODUCTION

Amino acids are organic compounds of biological importance comprise of two fundamental functional group i.e., amine and carboxylic acid along with a side chain specific to each amino acid[1], Tryptophan (Trp.) is an essential amino acid for humans and is considered exceptional in its diversity of biological functions[2]. It is a vital constituent of proteins and crucial in human diet for setting up a positive nitrogen balance[3], In particular, Trp. is the precursor of the neurotransmitter serotonin and plays an important role in brain function and related regulatory mechanisms[2,4]. In addition, Trp. is an important and frequently used starting material in the chemical synthesis of a range of pharmaceutical[5].

It has been used in the treatment of depression, Schizophrenia and hypertension[6] as well as some of its derivatives are potent drugs[7,8]. Trp. produced from proteins during digestion by the action of pro-

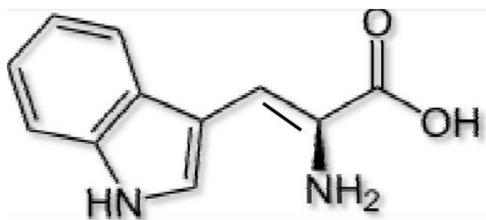


Figure 1 : Structure of tryptophan

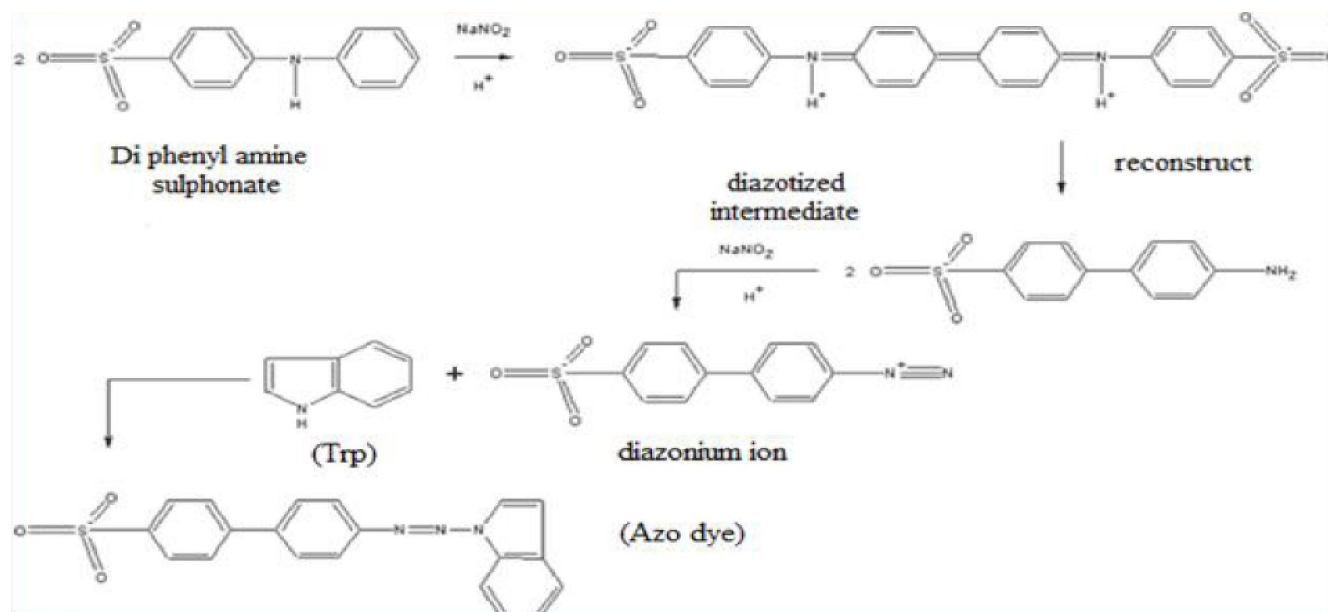
teolytic enzymes. It is a heterocyclic compound with $C_{11}H_{12}N_2O_2$ formula (Figure 1) and is present in small quantity in nearly all proteins[3] [(IUPAC name, 2- amino – 3 – (1H – indol – 3- yl) propanoic acid)]

It takes part in nourishment of in facts and in the biosynthesis of serotonin and niacin[9], for the reason that, serotonin is synthesized from the dietary. L-tryptophan made it crucial for brain functions and neuronal regulatory mechanism. Brain serotonin level is greatly influenced by an unbalanced of Trp. in diet[10]. Trp. is widely used in food industries to sustain the diet quality and added as a food fortifier and to balance the amino acid level. It can also be utilized to monitor the formation and dynamics of the proteins owing to its indol moiety[11]. Its nutritional and biochemical importance emphasizes the need for reliable analytical methods for the determination of Trp. in food and feed proteins[12].

Several approaches have been proposed for direct tryptophan determination or after derivatisation of Trp. content in biological media, in food or in pure form include voltammetry[13,14], titrimetry[15], capillary electrophoresis[16,17], polarography[18], amperometry[19,20], fluorescence spectroscopy[21,22], high – performance liquid chromatography[23-26], Chemiluminescence (CL)[4,27-30], spectrophotometric method[22,31-34], Flow injection analysis[4,28,30].

Among these methods, titrimetry[15] is the simplest but it is in sensitive. Although capillary electrophoresis[16] has attained substantial attention due to its high resolution but its operation is very complicated, voltammetric methods[13,18] are relatively inexpensive and sensitive. However the extracting qualitative and quantitative information from electrochemical data may result in a difficult task which is the main disadvantage of the voltammetric methods. HPLC method[23,24] is widely used for the determination of Trp. However the analysis of the Trp. remains problematic due to its lability to acid hydrolysis, so it is common to employ the sophisticated, time – consuming alkaline hydrolysis during the assay of Trp. by HPLC.

Even though Trp. have a luminescent chromophore, there is another fluorescing chromophore, tyrosine, in protein hydrolysates[21]. The spectrophotometric analysis is normally adopted because of relatively cheap and easy instrumentation but the previously reported spectrophotometric methods[22,31] also experiences several weaknesses such as call for time taking heat pretreatment steps, complicated extraction of samples to minimize the interferences



Scheme 1 : Reaction sequence mechanism

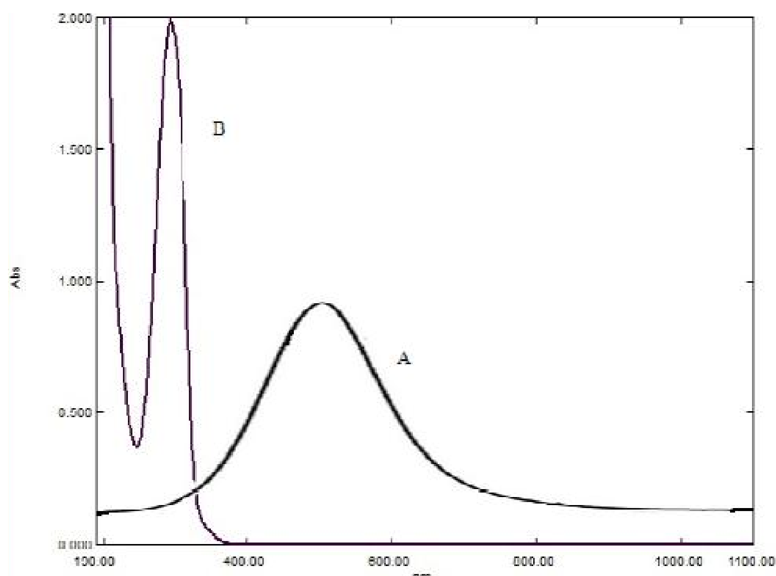


Figure 2 : (A) Absorption spectra of the azo dye 100 µg.ml^{-1} of tryptophan against reagent blank. (B) Blank against deionized water

of other chemical required. The object of the present study, we propose a new, quick, sensitive, economical, safe and sample methods to determine simultaneously and directly of Trp., in pure form and in pharmaceutical preparations via FIA / Merging zones techniques with spectrophotometric detection, the manifold consisted in one channel and six – three ways valves. The chemical process was proposed which involves an azo - coupling reaction of diphenylamine sulphonate with Trp. in sulphuric acid system to produce a pink color product with 522 nm of absorbance maximum. The proposed methods were designed in way that Trp., sulphamic acid, diazotized intermediate were simply loaded in FIA system based on the principle of merging zones through the homemade valves and deionized water as carrier(2.7 ml.min $^{-1}$) with no complicated extraction of samples, with no pretreatment, separation steps, time consuming and derivatisation reagents were avoided.

EXPERIMENTAL

Apparatus & manifold

All spectral and absorbance measurements were carried out on a UV-Visible -9200 (Shimadzu) digital double –beam recording spectrophotometer (Biotech Engineering management CO, LTD, (UK)) for batch procedure. and spectrophotometer SP-300 (Optima –Japan) for FIA procedure using a quartz flow cells with 50 µl internal volume and 1 cm bath length was used for the absorbance measurements. A one channel manifold, Figure (3) was used for the FIA spectrophotometric determination of Tryptophan. A Tubing peristaltic pump (two ways) (Master flex Permer,(USA)) was used to transport the reagents solution. Injection valve (six-three way plastic valve domestically made) which including three loops made of Teflon 0.5mm internal diameter that loaded with azotized product between DASA with sulphuric acid and sodium nitrite (L_1),Sulphamic acid (L_2), Tryptophan (L_3) based on merging -zones technique, were employed to provide appropriate injection volumes of standard solutions and samples. Reaction coil (RC) was made of glass with an internal diameter of 2 mm. Deionized water as carrier was combined with azotized product (L_1) and they merged with the sulphamic acid (L_2) and injected sample (Tryptophan with sodium hydroxide) (L_3), then mixed in reaction coil (RC) with length of 50 cm, injection sample of 43.175µl, flow rate of carrier of 2.7ml.min^{-1}, the absorbance was measured at 522 nm and at temperature (30^{0}C).

Chemical and reagents

All the chemicals used were of analytical grade & all the solutions were prepared with distilled

TABLE 1 : Optimum conditions established in batch method

Parameter	Optimum range	Conditions in procedure
λmax (nm)	350-600	522
Effect of H_2SO_4 concentration	0.1 –3M	2M
Effect of $NaNO_2$ concentration	$5x10^{-4}$ – $3x10^{-3}$ M	$2x10^{-3}$ M
Effect of DASA concentration	$1.25x10^{-4}$ – $7x10^{-4}$ M	$5x10^{-4}$ M
Effect of Sulphamic acid concentration	$2.5x10^{-4}$ – $6x10^{-3}$ M	$5x10^{-4}$M
Effect of temperature	0-45^{0}C	5^{0}C
Stability period after final dilution	1-200min	The colored product is formed right away and becomes stable after 1 min and remains for more than 1 hour

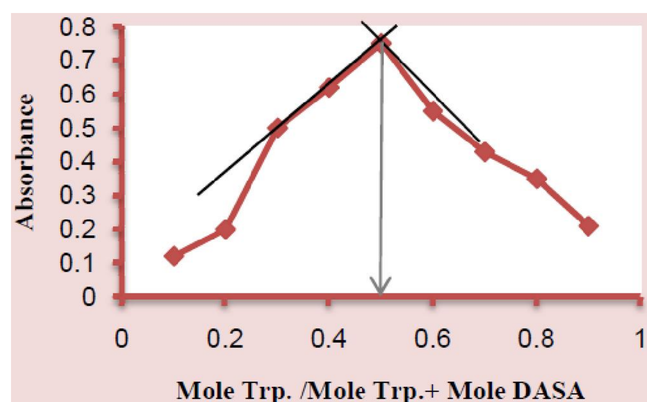


Figure 3 : Continuous variation plot of the reaction between trp. and diazonium ion

water, freshly prepared solutions were always used.

Tryptophan Stock solution (500µg.ml^{-1} = 2.4 x10^{-3}M)

A 0.05 g amount of pure Tryptophan (BDH) was dissolved in deionized water then completed to 100 ml in volumetric flask with deionized water More dilute solutions were prepared by suitable dilution of the stock standard solution with deionized water.

Diphenylamine sulphonate (1 x10^{-3}M)

An aliquot corresponding to 0.0271 g of DASA (BDH) was dissolved in 100ml volumetric flask with deionized water.

Sodium nitrite (2x10^{-3} M)

A (0.0138 g) amount of $NaNO_2$ (Merck) was dissolved in a 100 ml volumetric flask with deionized water

Sulfuric acid (BDH) (2M)

Was prepared by diluting 10.9 ml of 18.4M of concentrated Sulfuric acid (BDH) with deionized water in 100ml volumetric flask.

Sulphamic acid (SDI) (2x10^{-3} M)

Prepared by dissolving 0.0194 gm of sulphamic acid in deionized water and completed the volume to a 100ml in volumetric flask with deionized water, the solution stored in refrigerator avoiding direct light and used within one week.

Pharmaceutical preparations of Tryptophan (500µg.ml^{-1})

Pharmaceutical preparations were obtained from commercial sources.

1. Sundown Naturals, 5-HTP (L-5-hydroxy tryptophan), Dietary supplement 200mg, Supports a calm and Relaxed mode USA.
2. Noxidrim(5-HTP) Complement Alimentire 100mg (SOLGAR) USA.
3. Natural (5-HTP) TR Time Release USA 200mg, Dietary supplement.

To determine the content of 5-hydroxy tryptophan in capsules, the average weight were determined by selecting 13 tablets randomly from different packets 200,100mg. The tablets were weighted and the Hard gelatin capsules were removed and the contents were finely powdered then weighing an amount equivalent to 0.05g for each drug. The powder was dissolved in deionized water transferred into a 100ml volumetric flask, and completed to the mark with the same solvent. Then the solution was filtered to remove any insoluble residue affecting on the re-

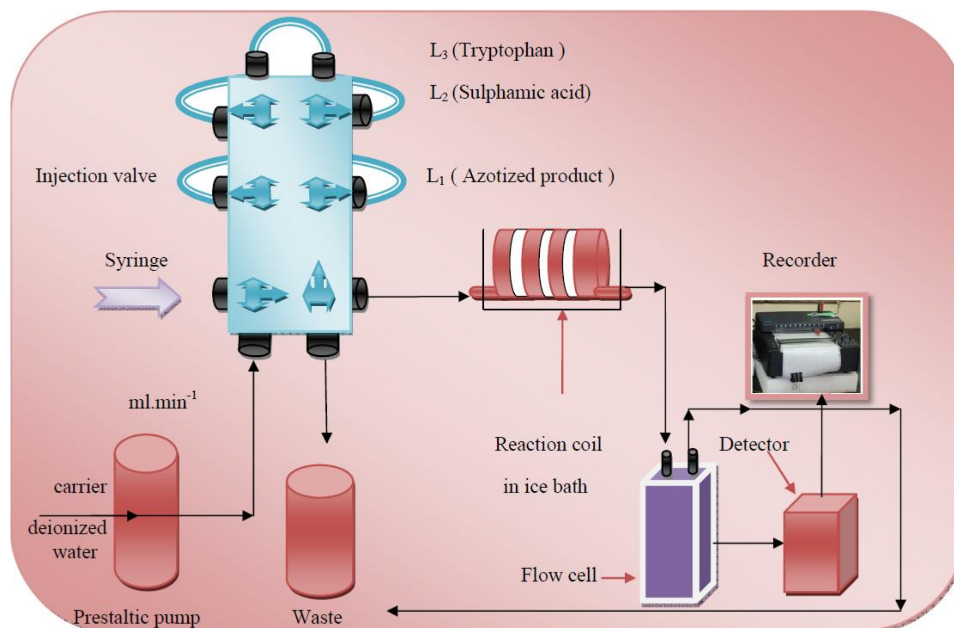


Figure 4 : A schematic diagram of FIA manifold

sponse. A further appropriate diluted solution were made up by dilution with deionized water.

MECHANISM OF THE REACTION

The mechanism of the reaction is azo - coupling. The reagent used is diphenylamine sulphonate which is oxidized to diphenylbenzidine sulphonic acid after reacting with sodium nitrite in the sulphuric acid medium. The unstable oxidation product reacts quickly with sodium nitrite to produce a diazotized intermediate. When the diazotized intermediate is coupled with tryptophan, a pink color product is developed, which is stable for at least 1 h at the ambient temperature. This colored product has the absorption maximum at 522 nm, as shown in scheme (1).

PROCEDURES

Batch procedure

A 3 ml of diphenyl amine sulphonate ($1x10^{-3}$ M) was transferred in to a series of 25 ml standard flask. Then the flasks were cooled in the ice bath to preserving a constant temperature of about 5^0C. A volume of 6ml of sulphuric acid was added in to each flask and mixed lightly. After 5min, 2.5ml of sodium nitrite ($2x10^{-3}$ M) were added and the mixture was placed in the ice bath for 5 min. A volume of 3 ml of sulphamic acid ($2x10^{-3}$ M) solution was added and cooled with shaking for further 5 min. The Tryptophan solutions were added to the flasks, the volumetric was made up to 25ml with sulphuric acid(2M), mixed and incubated for 15 min. The absorbance of the resulting pink-colored solution was scanned in the range of 450-600nm by a spectrophotometer (Shimadzu -9200), absorption maximum at 522 nm was recorded, as shown in Figure (2)

FIA procedure

Tryptophan solution in the range(5-100µg. ml $^{-1}$) was prepared from the standard working solution of 500µg.ml $^{-1}$. the injection volumes of (42.19,54.95,63.175 µl) of azotized product (Diphenyl amine sulphonate with sulphuric acid & sodium nitrite) (L_1), sulphamic acid ($2x10^{-3}$ M, L_2),and Tryptophan solution (5-100 µg.ml $^{-1,}$ L_3] respectively, were injected into the carrier of deionized water with flow rate 2.7 ml. min^{-1} as one channel, the resulting absorbance of the pink product conducted optimization of conditions were carried out on 100µg.ml $^{-1}$ of Tryptophan.

RESULTS AND DISCUSSION

Batch spectrophotometric determination

The factors affecting on the sensitivity and sta-

bility of the colored product resulting from reaction between azotized product and Tryptophan in acidic medium were carefully studied and optimized. A typical spectrum for the azo dye formed was measured against reagent blank which has negligible absorbance at λmax = 292 nm, as shown in Figure (2, A&B).

The experimental conditions for the determination of Tryptophan were performed through change one factors at a time and maintaining the other parameters fixed and observing the effects of the product on the absorbance.

The pink colored which was formed between tryptophan and azotized product had developed only in an acidic medium ; therefore, the effect of different concentration of acidic solution were studied. The maximum sensitivity and stability were obtained only when the reaction was carried out in the concentration of acid medium (2M) of H_2SO_4. The best experimental conditions for the determination of trp. were performed that H_2SO_4 (from 0.1 –3M), $NaNO_2$ (from $5x10^{-4}$– $3x10^{-3}$ M) and DASA (from $1.25x10^{-4}$ – $7x10^{-4}$ M), Sulphamic acid (from $2.5x10^{-4}$ – $6x10^{-3}$ M), the concentration of Tryptophan was 35µg.ml^{-1}. The colored product is formed right away and becomes stable after 1 min and remains for more than 1 hour. The effect of temperature on the color intensity of the azo dye was studied. A high absorbance was obtained when the color is developed at 5^0C. The optimum conditions for batch method were included in TABLE (1)

The stiocheiometry of the reaction between tryptophan and diazotized intermediate was investigated using continueous variation method (Job's method). The result obtained (Figure (3)) shows that a (1:1) azo dye formed between trp. and diazonium ion (scheme (1)).

The apparent stability constant was calculated by comparing the absorbance of solution containing stiochiometric amount of tryptophan($1.7x10^{-4}$ M) and diazotized DASA ($1.7x10^{-4}$M) (A_S) with that of a solution containing a five-fold excess of diazotized DASA reagent (A_m) and according to the batch procedure used. The average stability constant (K= $2.962x10^3$ l.mol^{-1}) where is

$[K=(1-\alpha) /\alpha^2C]$ & $\alpha = A_m-A_s/A_m$.

FIA spectrophotometric determination

The batch method for the determination of Tryptophan was adopted as a basis to develop a FIA procedure. The manifold used for the determination of Tryptophan was designed to provide different reaction conditions for magnifying the absorbance signal generated by the reaction of Tryptophan with

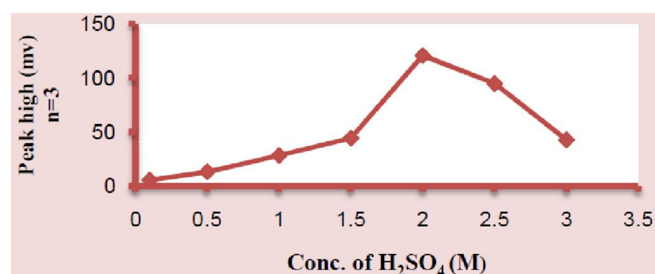


Figure 5 : Effect of the concentration of Sulfuric acid in (M)

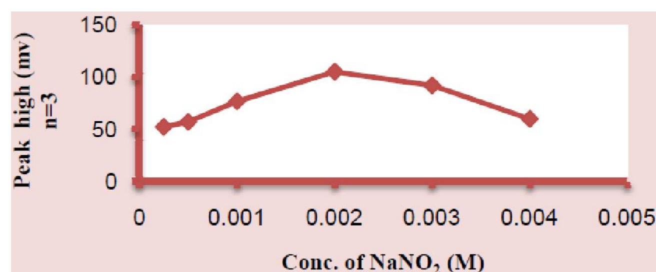


Figure 6 : Effect of the concentration of Sodium nitrate in (M)

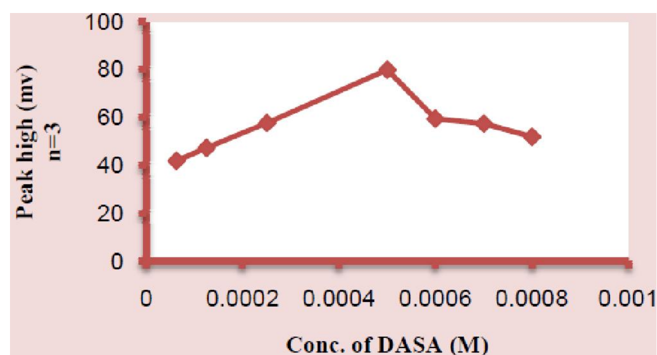


Figure 7 : Effect of the concentration of DASA in (M)

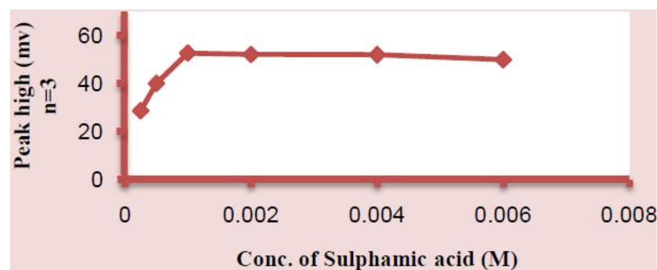


Figure 8 : Effect of Sulphamic acid concentration in (M)

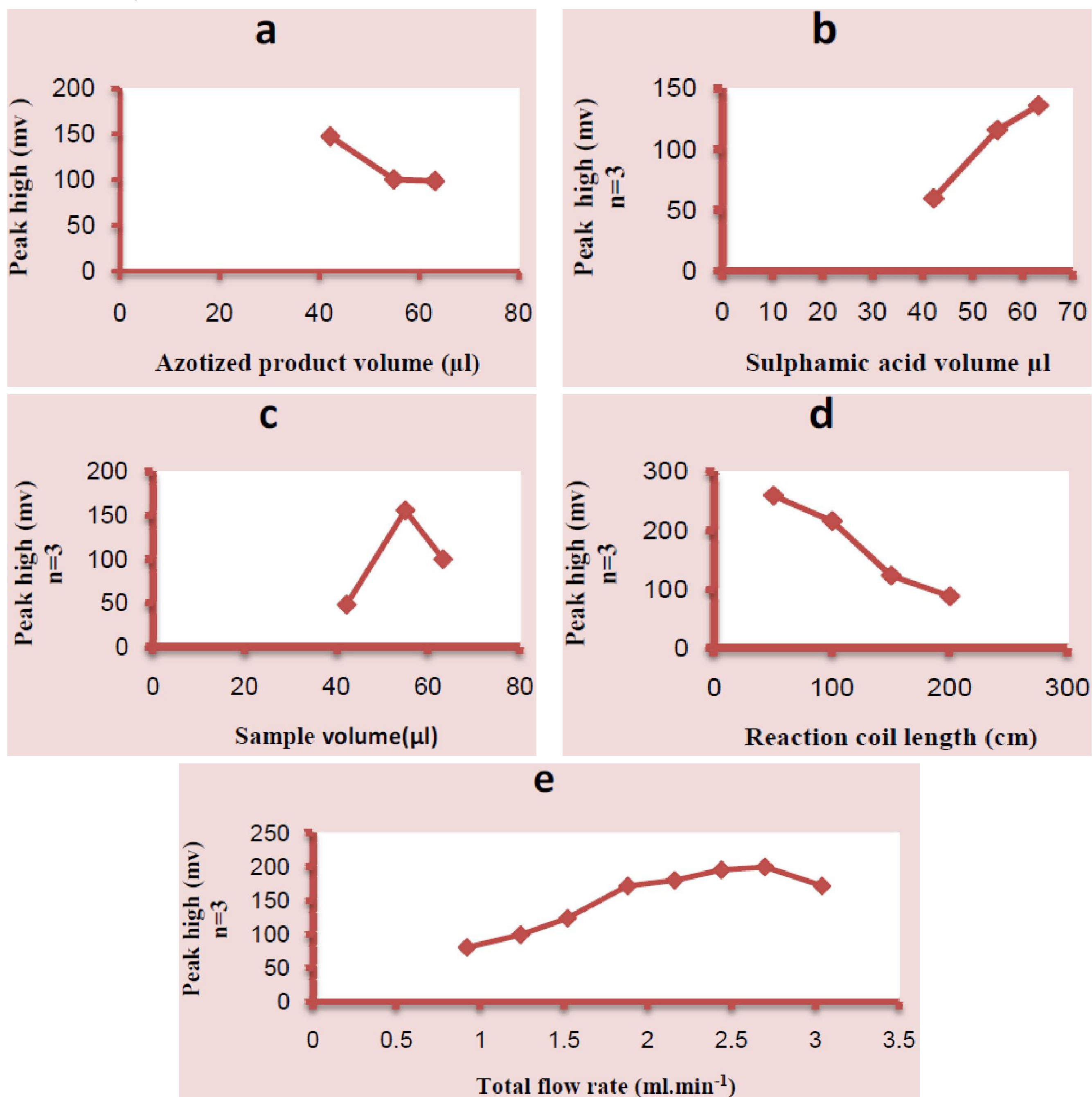


Figure 9 : Effect of manifold parameters on FIA (a) Effect of azotized product (b)Effect of sulphamic acid volume (c) Effect of sample volume (d) Effect of reaction coil length (e) Effect of total flow rate

azotized product in an acidic medium. Maximum absorbance intensity was obtained when the sample (100 µg.ml^{-1} of Trp.), Azo tized product (DASA ($5x10^{-4}$M), $NaNO_2$($2x10^{-3}$M), and Sulfuric acid (2 M)) and Sulphamic acid ($1x10^{-3}$M) were injected into a carrier of deionized water with flow rate of 2.7 ml.min^{-1}, as shown in Figure (4).

Optimization of chemical parameters

The effect of various concentration of Sulfuric acid (0.1 – 3 M) using as a medium was studied. It was found that(2 M) gave the highest absorbance and chosen for further experiments, as shown in Figure (5).

The effect of different concentration of sodium nitrate($2.5x10^{-4}$ – $4x10^{-3}$ M) was investigated. A concentration ($2x10^{-3}$ M) of sodium nitrate gave the highest response and was chosen for further experiments,

TABLE 2 : Analytical characteristics of the procedures developed for determination of tryptophan

Parameter	Batch procedure	FIA procedure
Regression equation	y = 0.024x+0.138	y = 2.866x+84.97
Linear rang ($\mu g.ml^{-1}$)	3-40	5-100
Correlation coefficient (r)	0.9970	0.9980
Linearity, (r^2%)	99.5	99.6
Relative standard deviation (RSD%)	0.547	0.58
Intercept, (a)	0.138	84.97
Slope, (b), ($ml.\mu g^{-1}$)	0.024	2.866
Standard deviation of intercept (S_a)	0.077	16.12
Standard deviation of slope (S_b)	$1.86x10^{-3}$	0.180
Confidence limit of intercept, ($a \pm ts_a$)	0.138±0.331	84.97±69.36
Confidence limit of slope, ($b \pm ts_b$)	$0.024 \pm 8.003x10^{-3}$	2.866±0.774
*Limit of detection ($\mu g.\ ml^{-1}$)	0.2325	0.188
**Limit of quantification	2.325	1.88
Sample through put (h^{-1})	4	52

***Limit of Detection = $3S_B$ /b, **Limit of quantification $3S_B$x10 / b.**

as shown in Figure (6)

A variable concentration of DASA in the range of ($6.25x10^{-5}$ – $8x10^{-4}$ M) was studied as shown in figure (7). The results obtained indicated that the high of peak was decreased with increasing the concentration of DASA ($5x10^{-4}$M) up to, thus a concentration of DASA ($5x10^{-4}$M) gave the maximum response, and was chosen for further use.

The effect of various concentration of sulphamic acid which was added to eliminate the remaining nitrate in the range ($2.5x10^{-4}$ – $6x10^{-3}$ M) was investigated. A concentration of ($1x10^{-3}$ M) of sulphamic acid gave the highest response and chosen for further experiments as shown in Figure (8)

Optimization of physical parameters

The physical variables under the optimized reagents were studied (flow rate, injection sample volume, Sulphamic acid volume, azotized product volume, Reaction coil length and purge time). The results showed that a flow rate 2.7ml.min^{-1} gave highest response and minimum dispersion figure (9-e) and it was used in all subsequent experiments. the volume of the sample, Sulpharic acid and azotized product was (42.19, 63.175, 54.95, µl) using different lengths of loop and showed that a sample (54.95µl),azotized product (42.19µl) and Sulpharic acid (63.175µl) gave the best response figure(9-a,b,c). moreover, a reaction coil length of (50cm) gave the highest response Figure (9-d) and was used in all subsequent experiments. A standard calibration graph, obtained from a series of Trp. standards and the main analytical of merits of the developed procedures are indicated and compared in TABLE (2)

ANALYTICAL APPLICATIONS

The suggested methods were applied to the analysis of some pharmaceutical preparations containing Tryptophan. Three type of pharmaceutical preparations were analyzed and they gave a good accuracy and precision as shown in TABLE (3). The obtained results indication clearly that there was no significant difference between developed method FIA with official method[35]. The results for dosage forms were compared statically by means the F- test and t-test at 95% confidence limits[36].

The calculated valued for F- test were (2.72) and (1.362), and t – test values were (0.3167) and (0.3491) for the batch and FIA methods respectively TABLE (4), did not exceed the critical values of F-test =19.009 and t-test = 2.770 (n_1+n_2-2=4). these indication that there are no significant differences

TABLE 3 : Application of the proposed methods to the determination of tryptophan in pharmaceutical preparations

Pharmaceutical preparation	Proposed methods	Conc. μg.ml^{-1} Present	Found	E %	*Rec%	*RSD%
Sundown Naturals 5-HTP (L-5-hydroxy tryptophan) Dietary supplement 200mg Supports a calm and Relaxed mode (USA)	Batch	15	15.06	+0.06	100.4	$7.8x10^{-4}$
		35	34.98	-0.02	99.942	0.0743
	FIA	20	20.15	+0.15	100.75	0.297
		50	49.91	-0.09	99.82	0.082
Noxidrim(5-HTP) Complement Aliment ire 100mg (Solcar) (USA)	Batch	15	15	0.0	100	0.0471
		35	34.99	-0.01	99.972	0.097
	FIA	20	19.99	-0.01	99.95	0.05
		50	50	0.0	100	0.014
Natural (5-HTP) TR Time Release (USA), 200mg, Dietary supplement	Batch	15	14.911	-0.089	99.41	1.72
		35	35.02	+0.02	100.057	0.148
	FIA	20	19.99	-0.01	99.95	0.1
		50	50.16	+0.16	100.32	0.558

*Mean of five measurements of each method

TABLE 4 : The comparison of the proposed method with official method

Pharmaceutical preparation	Proposed method Batch Rec%	Batch t*	Batch F*	FIA Rec%	FIA t*	FIA F*	Official method Rec%
Sundown Naturals 5-HTP (L-5-hydroxy tryptophan) Dietary supplement 200mg, (USA) Supports a calm and Relaxed mode	100.171			100.285			99.95
Noxidrim(5-HTP) Complement Aliment ire, (USA) 100mg (Solcar)	99.986	0.3167	2.72	99.975	0.3491	1.362	100
Natural (5-HTP) TR Time Release USA 200mg, Dietary supplement	99.733			100.135			100.2

Theoretical values at 95% confidence limit, $n_1=n_2=3$, t= 2.77 where t has $v= n_1+n_2-2$ degrees of freedom = 4, F= 19.099 where F has $v_1=n_1-1$, $v_2=n_2-1$ degrees of freedom = 2.

between the developed method and the official method in the determination of Trp. in pharmaceutical formulations,as shown below:

CONCLUSIONS

The proposed Homemade FIA/ merging zones methods are rapid and sensitive for the determination of Trp. with spectrophotometric detection. These methods can be used for the determination of μg. ml^{-1} amount of Trp. without the need for previous separation steps, temperature or pH control. The main advantages of the methods are its simplicity and its large dynamic range which make it possible to determine Tryptophan in the real samples with satisfactory results. The flow injection system developed for the determination Trp. is not expensive ; it employs available reagents, allows rapid determination at low operating cost, and provides simplicity, adequate sensitivity, and low limit of detection compared with the referenced methods. The procedures have good linearity, rapid, through –put 52 samples /h^{-1}. In addition, the wide applicability of the developed method for routine quality control is well established by analyzing the assay of Trp. at concentration of trace level (ppm) in pharmaceutical formulations. There is no significant differences between the proposed method and official method as shown in TABLE (4).

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