

Spectrophotometric Determination of Some Sulphonamide Drugs via oxidative coupling with Phenothiazine and Benzoyl peroxide using flow-injection technique

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Abstract

A novel flow injection Spectrophotometric method is described for the determination of microgram amounts of sulphonamides in aqueous and biological samples. The method is based on the oxidative coupling reaction of phenothiazine with sulphonamide drugs in the presence of benzoyl peroxide as oxidising agent. Intense bluish-green, water soluble stable dye was obtained which has a maximum absorption at 614 nm. Linear calibration graphs were obtained for different sulphonamides with a molar absorptivities of 3250-4720 $l \cdot mol^{-1} \cdot cm^{-1}$ and a relative standard deviation of better than 0.84 %. The proposed method has proved to be highly sensitive (detection limit range 0.30-0.63 $\mu g \cdot ml^{-1}$ sulphonamide) and accurate (recovery % was 97.9-100.2 %).

Optimum concentrations of chemical reactants, physical instrumental conditions and effects of interferences from certain additives have been investigated. The nature of the coloured produced dye and its stability constant were determined. The method was applied successfully for the determination of sulphonamide drugs in pharmaceutical preparations, blood and urine samples.

| | | | | | | | |
|------|------|--------|---------|--|---------|------|------|
| | | | | | | | |
| | | - | | | | 614 | |
| | | | | | | | |
| 0.63 | 0.30 | | . %0.84 | | 1- . 1- | 4220 | 3250 |
| | | %100.2 | 97.9 | | | | |

Introduction

Sulphonamides are widely used in the treatment of urinary tract infection burn therapy, conjunctivitis and chloroquine resistant malaria. They are also the drugs of choice for the treatment of nocardiosis, toxoplasmosis, severe travellers diarrhoea and meningococcal infections⁽¹⁾.

Various methods have been reported for the determination of sulphonamides. These include gravimetric⁽²⁾, titrimetric^(3,4), potentiometric⁽⁵⁾, amperometric⁽⁶⁾, polarographic⁽⁷⁾, chromatographic^(8,9), flame spectroscopy⁽¹⁰⁾, fluorimetric⁽¹¹⁾ and spectrophotometric methods⁽¹²⁻¹⁷⁾. Colorimetric methods based on diazotisation and coupling with phenols or arylamines such as N - (1 - naphthyl) ethylene diamine (Bratton and Marshall reagent). The latter seems to be the most popular method.

The object of the investigation reported in this paper was to evaluate an automated flow injection spectrophotometric method for the microdetermination of sulphonamide drugs in an aqueous medium using phenothiazine as a chromogenic reagent and benzoyl peroxide as oxidant. The application of the method for the analysis of drugs in blood, urine and pharmaceutical samples will also be considered.

Experimental

Apparatus and flow manifold:

Figure (1) shows the manifold used for the spectrophotometric determination of sulphonamides. The manifold consists of a peristaltic pump (P) of the type (Desaga Heidel Berg England) with six channels. A sample injection via a rotary valve (S) type Rehodyne U.S.A, with a sample loop was used to inject the sample volume into the flowing carrier

stream. Teflon tubes and mixing (reaction) coil (L) of 0.88 mm i.d were used in this flow manifold, also a home made (Y) piece (perspex) was used to mix two stream of reagents.

A spectrophotometric detector (D) type 4050 uv-visible beam linear readout provided with 60 μ l flowthrough cell. DC-microvoltmeter (UV) for increasing sensitivity a power supply were used as associated electronics and single pen x-t recorder (R) was used.

Reagents

All chemicals used were of analytical reagent grade. All sulphonamide drugs were obtained in a highly pure form and pharmaceutical preparations from the state drug industry (SDI) Sammara-Iraq. Standard Sulphonamide (500 μ g ml⁻¹) solutions: were prepared by weighing accurately 100 mg of sulpha drug, dissolved in 10 ml of absolute ethanol and diluted to 200 ml in a volumetric flask with distilled water.

Phenothiazine solution(1x10⁻³M): 0.01992 g of pure phenothiazine was dissolved in 100 ml of absolute ethanol. The solution was prepared daily and used⁽¹⁹⁾.

Benzoyl peroxide (2.5x 10⁻⁴M): 1.51250 g of benzoyl peroxide is dissolved in 250 ml of absolute ethanol.

Recommended procedures:

Procedure for calibration curve:

Take 5 \times 10⁻⁴ M benzoylperoxide 0.5 M H₂SO₄ and 6 \times 10⁻⁵ M of phenothiazine at flow rate 4 ml/min (Fig.1). Inject increasing volumes of sulphonamide working solution (100 μ g/ml). Measure the absorbance at 614nm against reagent blank, prepared in the same way but containing no sulphonamide using 1cm cells. In all subsequent experiments, 100 μ g/ml of sulphonilamide as sulphanilamide and sulphadiazine were used. The

absorbance is plotted against the final concentration to obtain a calibration curve.

Procedure for tablets :

20 tablets were finely powdered and weighed accurately. A portion of the powder equivalent to about 100 mg of the sulphonamide, was extracted with 10 ml of absolute ethanol. The solution was warmed and then, the mixture was filtered and washed with 5 ml ethanol. The combined filtrate and washings were diluted to 200 ml with distilled water (final concentration was $500 \mu\text{g ml}^{-1}$). 10 ml of this solution was diluted to 100 ml with distilled water to obtain $50 \mu\text{g ml}^{-1}$, 10 μl of the latter solution was injected into the phenothiazine line (Fig.1) using the optimum conditions, and the absorbance peak height was measured .

Procedure for prednisolone-s eye drops :

Five bottles of the prednisolone-s were mixed together and 1 ml of the prednisolone-s 10% sulphacetamide sodium (SAC) which contains 10 mg of sulphacetamide was mixed with 10 ml of ethanol and then diluted to a final volume 200 ml to contain a SAC. $50 \mu\text{g ml}^{-1}$ solution was prepared by dilution of 10 ml of $500 \mu\text{g ml}^{-1}$ to 100 ml with distilled water. The latter solution was injected into phenothiazine line (Fig.1).

Procedure for blood sample :

2 ml of serum was mixed with 30 ml of distilled water and 8 ml of 15% trichloro acetic acid, and then centrifuge for 5 min at a speed of 3000 rpm. and the supernatant liquid was filtered.

For the determination of free sulphonamide in the blood, the supernatant liquid was injected into the phenothiazine line (Fig.1). The absorbance peak height of the solution was recorded at 614 nm.

For the determination of total sulphonamide (free and conjugated), 0.5 ml of 2M HCl was added to 10 ml of the supernatant liquid (after precipitation of protein), then heated on a boiling water-bath for 1 hour. Cooled and adjusted to a volume of 10 ml, proceeding as for the determination of free sulphonamide .

Procedure for urine sample :

The urine sample was diluted five times with distilled water and the diluted urine solution was used for further experiments. For the determination of free sulphonamide in urine, 2 ml of the diluted urine was mixed with 30 ml of water and 8 ml of 15% trichloro acetic acid. The solution filtered and the supernatant liquid was injected into the phenothiazine line (Fig.1). The absorbance peak height of the solution was recorded at 614 nm.

For total sulphonamides (free and conjugated) after precipitation of protein, 5 ml of the diluted urine was placed in a 100 ml volumetric flask, 5 ml of 4 M HCl was added and the solution was diluted to the mark with distilled water, 10 ml of this solution was heated in a boiling-water bath for 1 hour. Cooled and adjusted the volume to a 10 ml, and the procedure was followed as for sulphonamides .

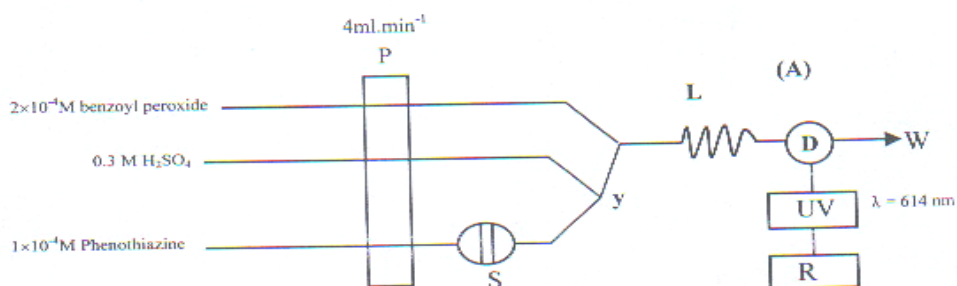


Fig.(1): Schematic diagram of the flow-injection manifold for determination of sulphonamides .

Results and Discussion

The reaction of sulphonamides with phenothiazine in the presence of benzoyl peroxide produces highly coloured bluish-green condensation product with a maximum absorption at 614 nm (Fig.2). The absorbance of the coloured product was measured using flow-injection analysis (FIA) system Fig.(1).

Initial studies were directed toward optimisation of the experimental conditions in order to obtain a good

sensitivity, stability and reproducibility of the coloured product. The influence of various reaction variables on the coloured product was tested to establish the most favourable conditions for the determination of $50 \mu\text{g ml}^{-1}$ sulphanilamide as an underivatised sulphonamide and sulphadiazine as an example of derivatised sulphonamide. Each sample was injected three times and the average peak height in mV is presented .

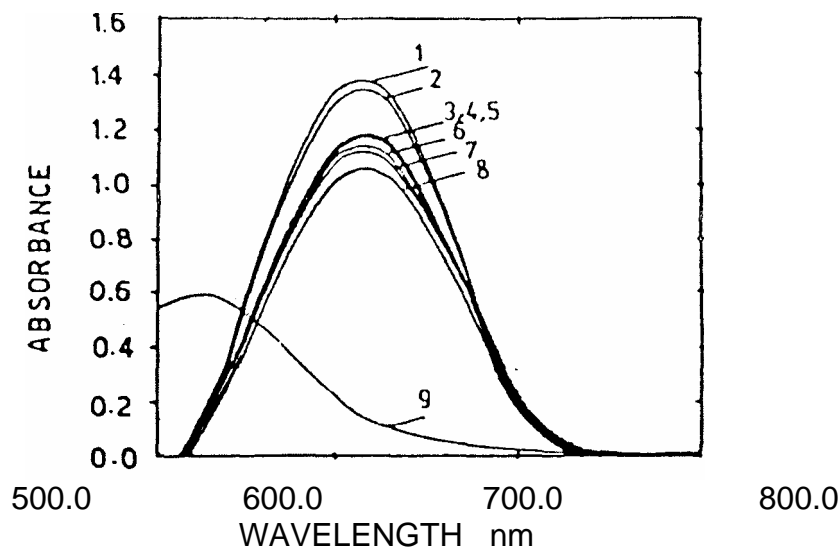


Fig.(2): Absorption spectra of the product obtained by the injection of $50 \mu\text{g ml}^{-1}$ sulphonamides into a stream of $1 \times 10^{-4} \text{ M}$ phenothiazine, is combined with 0.3 M H° and they mixed with $2 \times 10^{-4} \text{ M}$ benzoyl peroxide of: 1-Sulphacetamide sod., 2-Sulphanilamide; 3-Sulphaguanidine;4-Sulphathiazole; 5-Sulphamethoxazole; 6-Sulphadiazine; 7-Sulphamerazine;8-Sulphadimidine; 9-Absorption spectra of blank solution.

Optimization of conditions :**A- chemical optimizations:****Sulphuric acid concentration :**

Table (1) tabulates the variation of H_2SO_4 concentration (M) versus peak height (mV). A concentration of 0.3 M was found optimum and was used throughout the work .

Phenothiazine concentration :

Using optimised H_2SO_4 concentration at 0.3M and keeping all other chemical concentrations and physical parameters constant as indicated in the preceding section, effects of different phenothiazine concentrations are investigated. Table (2) tabulates the results, it is found that 1×10^{-4} M gives most sensitive result.

Benzoyl peroxide concentration :

Using optimised H_2SO_4 (0.3M),

phenothiazine (1×10^{-4} M) and other conditions mentioned in various sections, effect of different benzoyl peroxide concentrations are investigated. Table (3) tabulates the results, it is found that 2×10^{-4} M gave the best significant results.

B- Physical optimisations :**Length of delay reaction coil:**

Using the optimum concentration of reactants. Different lengths of delay reaction coil (190-280 cm) were tested.

Fig.3 shows that 235 cm coil length gives the best significant results. Longer reaction coil caused more sample dispersion⁽²⁰⁾. Longer diameter coil was avoided to prevent excessive dispersion reagent consumption⁽²¹⁾. Therefore 0.88 mm i.d. was selected and used through out the work.

Table (1) Effect of H_2SO_4 concentration* :

| Concentration of sulphuric acid (M) | Absorbance expressed as average (n=3) peak height in | |
|-------------------------------------|--|---------------|
| | Sulphanilamide | sulphadiazine |
| 0.0 | 0 | 0 |
| 0.1 | 54 | 51 |
| 0.2 | 126 | 120 |
| 0.3 | 160 | 153 |
| 0.4 | 150 | 142 |
| 0.5 | 135 | 129 |

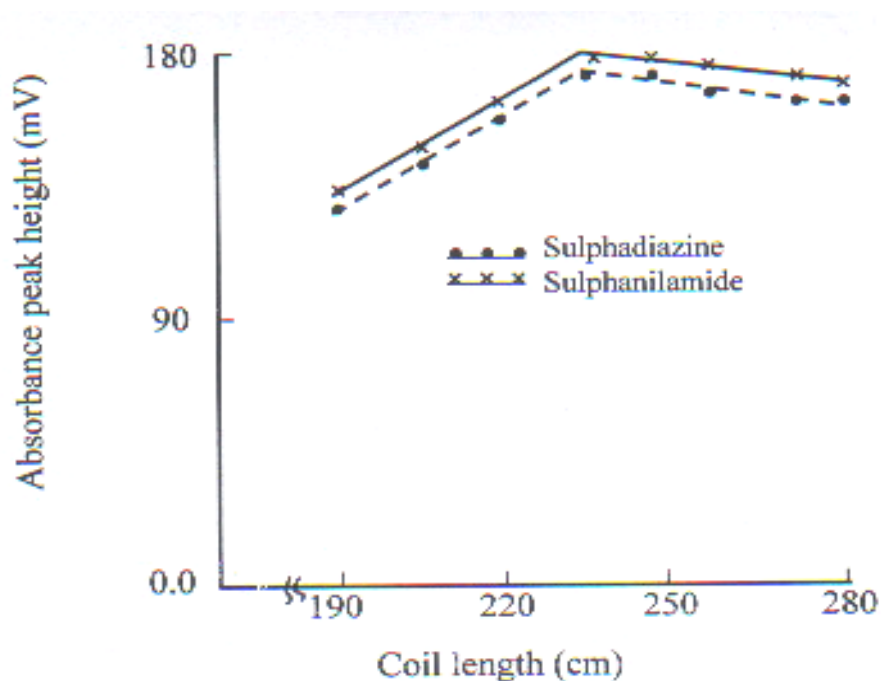
* Phenothiazine concentration 1×10^{-3} M, benzoyl peroxide 2×10^{-4} M. the system was operated at flow rate of 3 ml min^{-1} , reaction coil length was 220 cm, sample volume 60 μ .l at room temperature (25°C) and injection sample concentration $50 \mu\text{g ml}^{-1}$.

Table (2) Effect of phenothiazine concentration:-

| Concentration of phenothiazine | Absorbance expressed as average (n=3) peak height in mV of | |
|--------------------------------|--|---------------|
| | Sulphanilamide | sulphadiazine |
| 1×10^{-5} | 43 | 40 |
| 6×10^{-5} | 125 | 120 |
| 1×10^{-4} | 167 | 161 |
| 5×10^{-4} | 160 | 153 |
| 1×10^{-3} | 157 | 151 |

Table (3) Effect of benzoyl peroxide concentration:-

| Concentration of benzoyl | Absorbance expressed as average (n=3) peak height in mV | |
|--------------------------|---|---------------|
| | Sulphanilamide | sulphadiazine |
| 2×10^{-5} | 36 | 32 |
| 5×10^{-5} | 84 | 80 |
| 1×10^{-4} | 167 | 161 |
| 2×10^{-4} | 173 | 165 |
| 4×10^{-4} | 160 | 156 |
| 2×10^{-3} | 108 | 103 |

**Fig (3): Effect of coil length.**

Flow-rate :

The FIA system (Fig.1) was operated at different flow rates (2-10 ml min⁻¹), and it was found that 4 ml min⁻¹ is the optimum flow-rate (Fig.4).

Sample volume:

Different sample volumes (60-180 µl) were tested. The results obtained (table 4), showed that 60 µl is the optimum sample volume, and was used throughout the work.

Effect of temperature :

Table 5 indicated that the absorbance of the coloured product was decreased when the reaction was carried out at 0 °C , this may be due to the decrease in the reaction rate by decreasing the temperature, but when the reaction was carried out at 50 °C, the absorbance also decreased, which probably due to partial decomposition of the product. Therefore, the reaction mixture should be carried out at room temperature (25°C). The optimum working conditions were tabulated in table 6.

Calibration graph :

The results for the calibration graphs of sulphonamides obtained under the optimised conditions using the FIA manifold are illustrated in Table 7. Intense blue-green, water soluble stable dye was obtained which has a maximum absorption at 614 nm it appear from the results that the slopes of the calibration graphs vary considerably. This is probably due to the difference in the reaction

rate and different substituents of each sulphonamide, therefore, individual working calibration graphs are required and so that the method can be applied only to samples containing one sulphonamide. Sulphanilamide, the simple molecule, has the fastest reaction, probably because the other sulphonamides are sterically hindered⁽²²⁾. A t-test are conducted for linearity relation and it shows that $t_{cal.} = 620.17$ compared with $t_{tab.} = 2.23$ at 95% confidence limit which indicates rejection of H_0 (non linearity) against alternative hypothesis (linearity). The linear regression equation for the range of concentration 0.7-75.0 µg ml⁻¹ sulphanilamide at 95% confidence limit is Abs. (av. Pk. hgt. (n=3)) mV = (4.347 ± 0.513) + (3.476 ± 0.016) [sulphanilamide] µg ml⁻¹.

Precision and accuracy :

Using the optimum conditions (Table 6), the precision for the determination of sulphonamides by FIA were studied. Table 8 shows the relative standard deviation(R.S.D) for five injections of each sulphonamides containing 10,30,50 µg ml⁻¹ concentrations.

For estimation the accuracy of the method, five injections were performed containing 30 µg ml⁻¹ of each of the sulphonamide of the same solution investigated. The results obtained indicated that the average range between 97.90% and 100.20%.

Table (4) Effect of sample volume:-

| Sample Volume(µl) | Absorbance expressed as average (n=3) peak height in mV | |
|-------------------|---|---------------|
| | Sulphanilamide | Sulphadiazine |
| 60 | 173 | 165 |
| 80 | 161 | 154 |
| 100 | 154 | 148 |
| 120 | 143 | 137 |
| 130 | 135 | 129 |
| 140 | 130 | 126 |
| 180 | 123 | 120 |

Table (5) Effect of temperature:

| Temp.(°C) | Absorbance expressed as average (n=3) peak height in mV of | |
|-----------|--|---------------|
| | Sulphanilamide | Sulphadiazine |
| 0 | 150±1.23 | 143±1.07 |
| 10 | 162±1.65 | 149±1.11 |
| 15 | 165±1.24 | 158±1.25 |
| 25 | 173±1.02 | 165±1.10 |
| 30 | 161±1.50 | 148±1.33 |
| 40 | 150±1.23 | 140±1.55 |
| 50 | 141±1.49 | 133±1.63 |

*at 95% confidence limit.

Table (6) Optimum working condition for spectrophotometric determination of sulphonamide by FIA system using phenothiazine and benzoyl peroxide:-

| parameter | Value |
|--------------------------------|-------------------------------|
| Flow rate | |
| Reaction coil length | 4ml min ⁻¹ |
| Sample Volume | 235cm |
| Sulphoric acid concentration | 60 µl |
| Phenothiazine concentration | 0.3M |
| Benzoyl peroxide concentration | 1x10 ⁻⁴ M |
| Wave length selected | 614nm |
| Temperature | 25 ⁰ C(room temp.) |
| Reaction tubing diameter | 0.88 mmi.d |

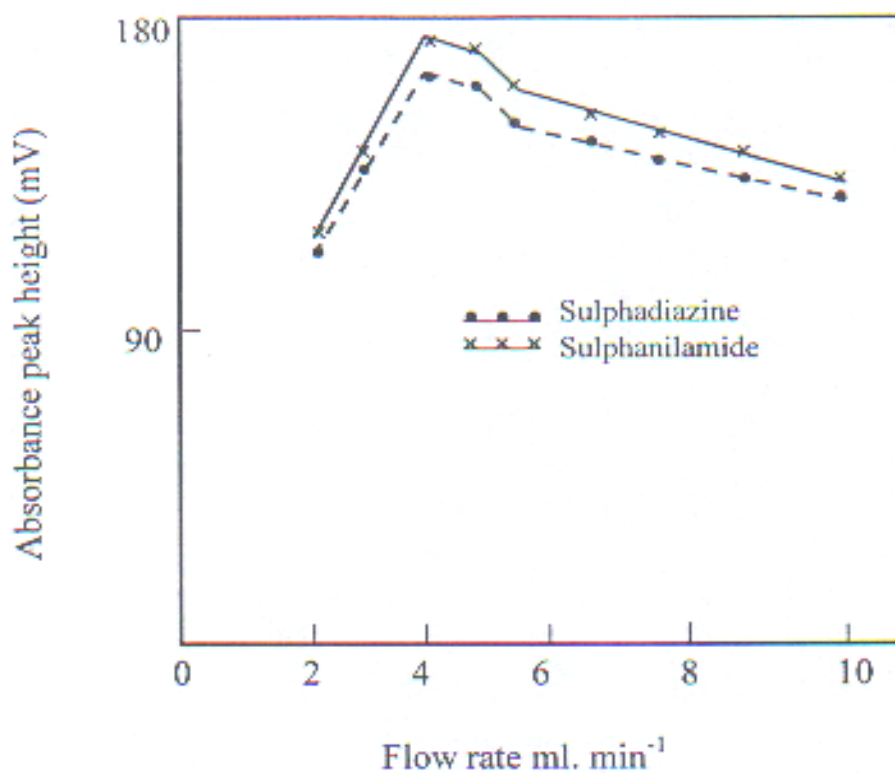
Table (7) Spectral characteristics of the products from the reaction of sulphonamides with phenothiazine and benzoyl peroxide by automated FIA:-

| Sulphonamides | Detection limit (µg ml ⁻¹) | Σmax (l. mol ⁻¹ . cm ⁻¹) | Linear range (µg ml ⁻¹) | Intercept (mV) (a±tSa) | Slope (mm.ml . µg ⁻¹) (a±tSa) | Correlation coefficient (r) |
|--------------------|--|---|-------------------------------------|------------------------|---|-----------------------------|
| Sulphanilamide | 0.30 | 3.30x10 ³ | 0.7-75.0 | 4.347±0 | 3.476±0.01 | 0.9999 |
| Sulphadiazine | 0.52 | 3.35x10 ³ | 0.7-100.0 | 4.113±0 | 3.178±0.02 | 0.9996 |
| Sulphaguanidine | 0.58 | 3.43x10 ³ | 2.0-90.0 | 4.036±0 | 3.265±0.01 | 0.9997 |
| Sulphadimidine | 0.46 | 3.25x10 ³ | 1.0-75.0 | 4.157±0 | 3.283±0.01 | 0.9993 |
| Sulphacetamide sod | 0.33 | 4.72x10 ³ | 0.7-100.0 | 4.259±0 | 3.093±0.25 | 0.9996 |
| Sulphamerazine | 0.63 | 3.37x10 ³ | 1.0-100.0 | 4.538±0 | 3.642±0.01 | 0.9999 |
| Sulphamethoxazole | 0.56 | 3.42x10 ³ | 2.0-100.0 | 4.231±0 | 3.356±0.01 | 0.9998 |
| sulphathiazole | 0.42 | 3.45x10 ³ | 2.0-90.0 | 4.623±0 | 3.216±0.01 | 0.9997 |

* at 99% confidence limit.

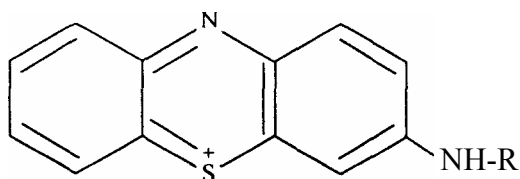
Table (8) Precision of the method:

| Compound | R.S.D % | | |
|---------------------|--------------------------|--------------------------|--------------------------|
| | 10 $\mu\text{g ml}^{-1}$ | 30 $\mu\text{g ml}^{-1}$ | 50 $\mu\text{g ml}^{-1}$ |
| Sulphanilamide | 0.84 | 0.49 | 0.34 |
| Sulphadiazine | 0.52 | 0.32 | 0.00 |
| Sulphaguanidine | 0.43 | 0.40 | 0.20 |
| Sulphadimidine | 0.67 | 0.51 | 0.30 |
| Sulphacetamide sod. | 0.30 | 0.25 | 0.10 |
| Sulphamerazine | 0.51 | 0.43 | 0.22 |
| Sulphamethoxazole | 0.43 | 0.38 | 0.11 |
| sulphathiazole | 0.47 | 0.31 | 0.23 |

**Fig.(4): Effect of Flow rate.****Nature of the product:**

The mole-ratio method for the monitoring of stoichiometry of the products was adopted to flow techniques.

The results (Fig.5&6) proved the existence of 1:1 sulphonamide : phenothiazine and the structure of the dye may be written as follows :



The stability constant ($K_{stab.}$) can be calculated using the following equation⁽²³⁾:

$$K_{stab.} = \frac{a - (\Delta A / \epsilon)}{n^2 (\Delta A / \epsilon)^{n+1}}$$

Where a = concentration of sulphonamide.

ϵ = molar absorptivity

n = mole ratio.

$\Delta A = A_m - A_s$

A_m = absorbance of a solution containing sulphonamide five times excess amount of phenothiazine.

A_s = absorbance of a solution containing a stoicheometric amounts of sulphonamide and phenothiazine.

The conditional stability constant of different sulphonamide products are summarised in Table 9.

Analytical applications :

Sulphonamides are usually formulated in

tablet form, therefore the effect of common tablet excipients and additives on the procedure was investigated. It was found that glucose, lactose, sucrose, starch and magnesium stearate had no effect on the assay (Table 10).

The effect of other drugs which are commonly prescribed with sulphadiazine, e.g., chloramphenicol, phthalyl sulphathiazole and succinyl sulphathiazole were also investigated and it was found that none of these drugs interferes in the method.

(Table 11) shows the results obtained from the determination of sulpha drugs in some of their dosage forms by means of the proposed method, these data indicate that the method has a good recovery (99.4-99.8%), except trimethoprim (147.0% recovery) since it contains an additional primary aromatic amine. A satisfactory result (Table 12) was obtained for the analysis of blood and urine samples.

Table (9) The stability constant of the products obtained by the reaction of sulphonamides with phenothiazine and benzoyl peroxide using FIA system:-

| Compound | Stability constant l. mol ⁻¹ |
|---------------------|---|
| Sulphanilamide | 2.52x10 ⁶ |
| Sulphadiazine | 2.61X10 ⁶ |
| Sulphaguanidine | 2.74x10 ⁶ |
| Sulphadimidine | 2.45x10 ⁶ |
| Sulphacetamide sod. | 5.28x10 ⁶ |
| Sulphamerazine | 2.64x10 ⁶ |
| Sulphamethoxazole | 2.72x10 ⁶ |
| sulphathiazole | 2.77x10 ⁶ |

Table (10) Effect of excipients an additiveson the recovery of sulphonamide drugs FIA-phenothiazine-benzoyl peroxide method.

| Excipient | Amount add µg ml ⁻¹ | Amount add to know the fold excess | Amount per 10 mg of drug/mg | Sulphanilamide* | | Sulphadiazine* | |
|-----------|-----------------------------------|---|--------------------------------|-----------------|--------|----------------|--------|
| | | | | Recovery % | R.S.D% | Recover y% | R.S.D% |
| Glucose | 50 | 80 | 0.5 | 99.6 | 0.51 | 100.5 | 0.70 |
| Lactose | 20 | 50 | 2.0 | 99.7 | 0.53 | 99.4 | 0.73 |
| Sucrose | 50 | 70 | 0.5 | 100.4 | 0.54 | 99.7 | 0.69 |
| Starch | 20 | 40 | 2.0 | 99.8 | 0.62 | 99.7 | 0.78 |
| Magnesium | 20 | 70 | 0.5 | 9.5 | 0.57 | 100.2 | 0.80 |

*average of five determinations.

Table (11) Mean recovery of sulpha drugs in dosage form by FIA - phenothiazine-benzoyl peroxide procedure:-

| Compound | Amount mg or ml per tablet Found by | | Mean recovery% |
|--------------------------------|--|----------------|----------------|
| | Nominal | present method | |
| Methoprim tablets ^x | 400 | 588 | 147.0 |
| Sulphaguanidine tablets | 500 | 497 | 99.4 |
| Sulpadimidine tablets | 500 | 499 | 99.8 |
| Prednisolone*-S eye drops | 500 | 498 | 99.6 |

+ average of five determinations .

^x Each tablet contains 400 mg of sulphamethoxzole and 80 mg of trimethoprim. Few tablets crushed, sieved then weight a known amount equivalent to one tablet weight, because no single tablet contain the same amount of active ingredient.

* Each 100 ml contain 10 g sulphacetamide sodium and 0.25 g of Prednisolone-S The Darow Co Tehran-Iran.

Table (12) Assay of sulphonamide drugs in blood and urine using FIA-phenothiazine-benzoyl peroxide procedure.

| Sample | Drug administered | Amount found µg ml ⁻¹ | |
|--------|-------------------|----------------------------------|--------------|
| | | Free | Total |
| Blood | Sulphanilamide | 0.974±0.019 | 1.135±0.033 |
| Blood | Sulphadiazinee | 0.864±0.022 | 1.027±0.024 |
| Urine | Sulphanilamide | 1.462±0.035 | 2.108±0.040 |
| Urine | Sulphadiazinee | 1.415±0.031 | 2.201 ±0.045 |

* For 95% confidence limit (n=3) at $U = X \pm 1 \frac{\sigma_{n-1}}{\sqrt{n}}$

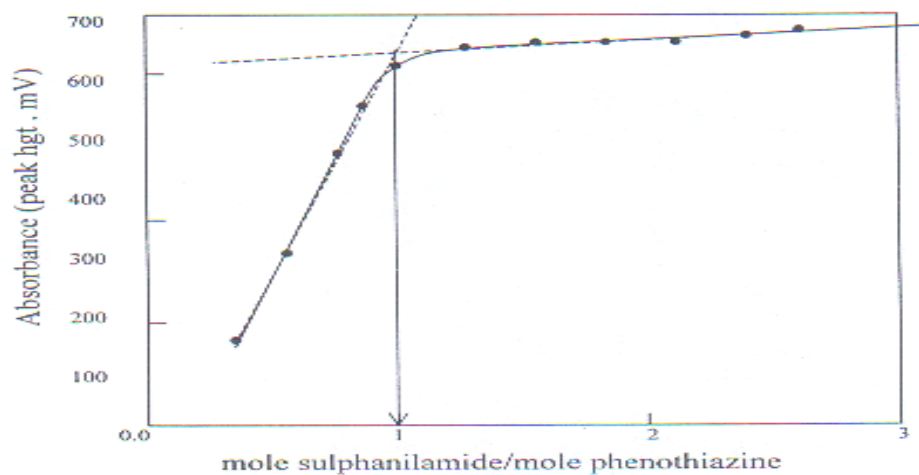


Fig.(5) Mole-ratio plot of the sulphanilamide-phenothiazine product

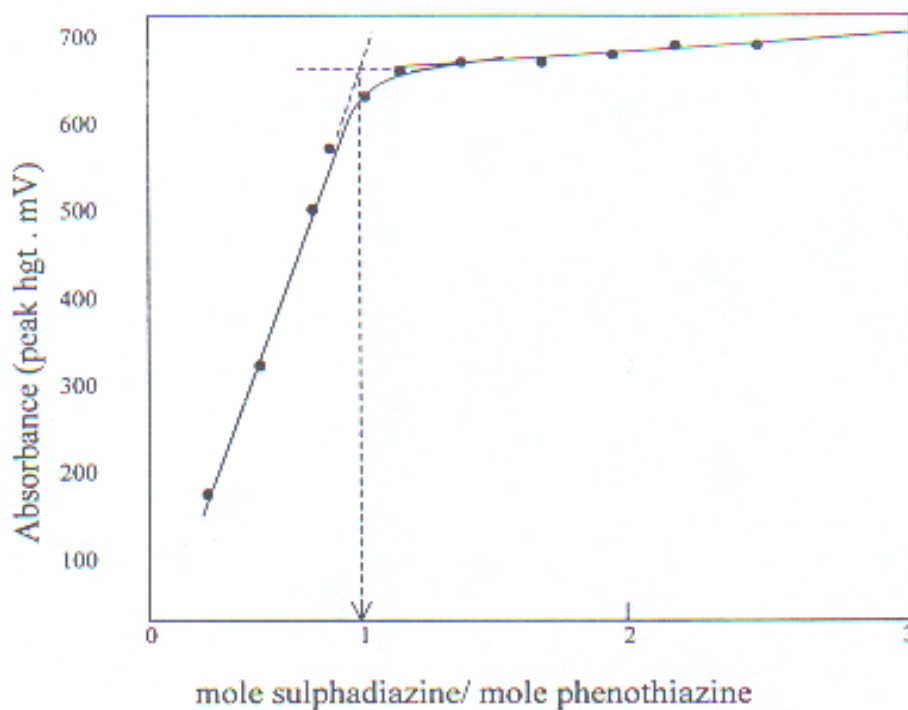


Fig.(6): study nature of the sulphadiazine – phenothiazine Product.

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