

Research Article

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Development and validation of the simple and sensitive spectrophotometric method of sulphanilamides determination with 4-(2-thiazolylazo)-resorcinol in veterinary preparations

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Abstract: New simple, rapid, and accurate spectrophotometric techniques for sulphanilamides assay in combination drugs have been elaborated. The method is based on the formation of coloured products of sulphanilamides diazonium salt azocoupling with heterocyclic azo reagent 4-(2-thiazolylazo)-resorcinol (TAR) in alkaline medium (pH 9.5) with the following measurement of their absorbance at $\lambda_{\text{max}} = 615 \text{ nm}$. Formed products are stable for 1 hour at room temperature. Effective molar absorptivities at absorbance maximum 615 nm for disazo dyes were $\sim 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. Linear ranges for sulphanilamides determination were 0.5–30.0 $\mu\text{g mL}^{-1}$. The developed method was used for the suphanilamides determination in tablets, powder, solutions, suspensions and ointments.

Keywords: Sulphanilamides, 4-(2-thiazolylazo)-resorcinol, Spectrophotometry, Diazotization, Azocoupling, Medicines.

1 Introduction

A heterocyclic azo reagent in analytical chemistry is mainly used for determination of metals ions. In the analysis of organic substances, especially in pharmaceutical analysis, they rarely are employed. One of the simplest members of this class of reagents is 4-(2-thiazolilazo)-resorcinol (3-hydroxy-4-(1,3-thiazol-2-ylhydrazono)cyclohexa-2,5-dien-1-one; 2-(2, 4-(dihydroxyphenylazo) thiazole) (dark red powder; easily soluble in water) (Table 1). This azo reagent can exist in three forms depending on the acidity in aqueous solutions, i.e., protonated on the nitrogen atom in the heterocycle (cationic form), molecular, and ionized by hydroxyl groups of resorcinol ring. TAR is a colored compound with the absorbance maximum of its solution in the visible portion of the spectrum. Changing the existence form of the reagent is reflected by a significant shift (85 nm) of the maximum absorbance band to the long-wavelength range of the spectrum at the transition from protonated (cation-) to molecular as well as dissociated forms. One-ionized azo reagents can exist in azo- or quinone-hydrazo-form, which is reflected in their reactivity (Scheme 1).

TAR is widely known in inorganic analysis as a spectrophotometric reagent for the spectrophotometric determination of platinum [8] and transition [9-11] metals. TAR was used in ion-exchange liquid [12-16] and thin-layer [17] chromatography for the determination of metals ions in natural and drinking water as well as for electrophoretic determination of metals [18-20], in optical [21-23] and ion-selective [24,25] sensors to determine the metals ions, for obtaining of the sorbents modified by azo reagent for concentration metals ions [26-30].

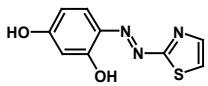
In the analysis of organic substances use of TAR is limited. A spectrophotometric method of the unbound fatty acid determination was described; it is based on the

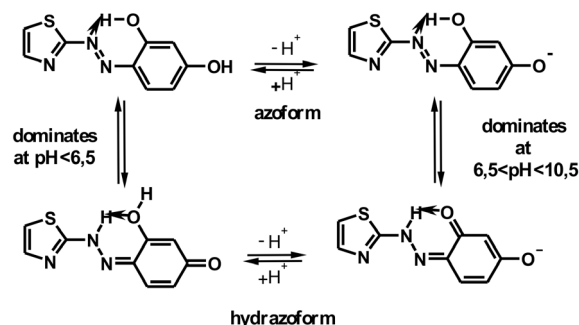
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Table 1: Structural formula and some characteristics of azo compound TAR [1-7].

Structural formula	Characteristics
CAS № 2246-46-0 	$pK(H\text{Ind}^+) = 1,0$; $pK(H^3\text{Ind}) = 6,2$; $\epsilon = 2,22 \times 10^4$, pH = 4,8; $pK(H\text{Ind}^-) = 9,3$; $\epsilon_{430} = 3,05 \times 10^4$, pH = 9,8; Ind^{2-} $\epsilon = 3,75 \times 10^4$, pH = 13,2 ₅₁₅

**Scheme 1:** Existence forms of TAR in aqueous solutions [1-7].

formation of the ternary complex of Cu (II), TAR, and fatty acid which allows for determining the latter in nanomolar quantities [31].

We used this reagent for spectrophotometric determination of sulphanilamides (SA), antibacterial drugs, which are widely used in veterinary practice as a combination drug with antibiotics and other antibacterial compounds. The SA determination in a combination drug is carried out mostly by the chromatographic analysis [32-35], since other methods (titrimetric, electrochemical, spectrophotometric) do not allow selective determination of the SA content in complex medicinal mixtures.

Numerous organic reagents including different dyes are used for SA determination by spectrophotometric method. In particular using of alizarin and its derivatives is known [36]. At our laboratory we have previously developed the techniques of SA determination using the mono azo dye Tropaeolin O [37,38] and heterocyclic azo reagent 4-(2-pyridylazo) resorcinol [39,40] as well as *o,o'*-dihydroxysubstituted azo dyes eriochrom blue SE [41], eriochrom blue-black R [42] and eriochrom black T [43]. The aim of this research is to establish the conditions of SA interaction with another heterocyclic azo reagent 4-(2-thiazolylazo)-resorcinol for obtaining of maximum analytical signal and the development of method of SA quantitative determination with satisfactory validation characteristics.

2 Experimental procedure

2.1 Reagents and apparatus

All aqueous solutions were prepared using distilled water.

Sulphanilamides were purchased from Sigma (USA). Solutions of sulphamethoxazole, sulphamethazine, sulphamerazine, sulphadimetoxine, sulphathiazole, sulphadiazine, sulphametoxy-pyridazine, sulphamonometoxine were prepared by dissolving appropriate amounts of the reagents of pharmacopoeia grade in 0.1 M sodium hydroxide solution. Solutions of sulphanilamide were prepared by dissolving appropriate amounts of the reagents of pharmacopoeia grade in 0.1 M hydrochloric acid. Solutions of sulphaguanidine were prepared by dissolving appropriate amounts of the reagents of pharmacopoeia grade in the mixture of equal amounts of 0.1 M hydrochloric acid and 96% ethanol. All solutions were stored at a room temperature in a dark place.

Solutions of TAR (Merck, Germany) were prepared by dissolving appropriate amounts of the reagent of analytical grade $\geq 98\%$ purity in 0.1 M sodium hydroxide solution.

The solutions of hydrochloric acid, boric acid, phosphoric acid, acetic acid, sodium hydroxide, sodium nitrite, sodium tetraborate, sodium acetate, sodium sulphate, sodium nitrate, sodium phosphate, sodium pyrophosphate, sodium carbonate, ammonium chloride, ammonium hydroxide, potassium bromide, calcium chloride, and magnesium chloride were prepared from the chemicals of the analytical grade.

UV-VIS measurements were performed with UV-VIS scanning spectrophotometer CARY.WIN – UV-VIS-50 (Varian, USA) and SPECORD M-40 (Carl Zeiss Jena, Germany) using 1 cm cuvettes. All absorbance measurements were performed at 20–25°C.

The pH value was measured by pH-meter RV 11 (Sartorius, Germany) as well as pH-meter model pH 150M (Gomelsky Plant of Measuring Devices, Belarus), equipped with a combination electrode, which incorporates both glass and reference silver chloride electrodes into one body. The required pH of each solution was adjusted using diluted HCl and NaOH solutions.

Polarographic researches were performed using an oscillograph PO-5122 model 03 (Russia) with additional computerised equipment and a three-electrode thermostated cell with an indicator mercury dropping electrode, auxiliary platinum electrode and the reference saturated calomel electrode (linear potential range from

-0.2 to -1.75 V by the defined conditions: potential sweep rate – 2.0 mV s⁻¹, the delay imposition voltage – 4.0 sec). Polarograms were recorded at a room temperature (20–25°C). Dissolved oxygen was eliminated from the test solutions by means of bubbling with purified argon for 15 min.

2.2 Procedure of finished dosage forms preparation for the SA determination

Procedure of tablets or powder preparation for the SA determination

Twenty tablets of every examined drug produced in dosage form of tablets were weighed and finely powdered in a porcelain mortar. The accurate amount of powder, containing ~ 100 mg of SA, was placed into a 100 mL volumetric flask and was dissolved in 50 mL of 0.1 M NaOH for obtaining SA extract. Then solution was mixed for 10 min and 0.1 M NaOH was added to complete the volume of 100 mL. Obtained solution was mixed again and was filtered through the fold filter of medium porosity. The filtrate was 10-fold diluted. Furthermore 5.0 mL of filtrate was placed into a 50 mL volumetric flask and diluted by 0.1 M sodium hydroxide solution to the full volume of 50 mL. Nominal SA content in solution obtained in such way was 100 µg mL⁻¹. For assay solution an aliquot of 1 mL was picked out and then treated as described below in the recommended procedure for the sulphanilamides determination.

Procedure of suspension or ointment preparation for SA determination

Sample of suspension or ointment containing 100 mg of SA in accordance with the specified content in drug, was placed into a chemical glass and 50 mL of 0.1 M sodium hydroxide was added. Mixture was heated on a water bath at the temperature of ~ 80°C for at least 10 min to complete melting of the ointment. Received mixture was filtered through a folded paper filter (white ribbon) in a volumetric flask 100 mL (the glass with mixture was kept in hot water bath to prevent solidification of the preparation till filtering was complete). Then the filter was washed several times with hot solution of 0.1 M sodium hydroxide. The filtrate was cooled and the content of the flask was filled up to the mark on the flask with the same solvent and was used for analysis. The filtrate was 10-fold diluted. Thereto 5.0 mL of filtrate was placed into a 50 mL volumetric flask and diluted by 0.1 M sodium hydroxide solution to the full volume of 50 mL. Nominal SA content in the solution obtained in such way was 100 µg mL⁻¹. For

assay solution an aliquot of 1 mL was taken and then was treated as described below in the recommended procedure for the sulphanilamides determination.

Procedure of solution preparation for the SA determination

Aliquot of solution containing 100 mg of SA was placed into a 100 mL volumetric flask and 50 mL of 0.1 M NaOH was added for SA extract obtaining. Then solution was mixed for 10 min and 0.1 M NaOH was added to complete the volume of 100 mL. The filtrate was 10-fold diluted. Thereto 5.0 mL of filtrate was placed into a 50 mL volumetric flask and was diluted by 0.1 M sodium hydroxide solution to the full volume of 50 mL. Nominal SA content in solution obtained in such way was 100 µg mL⁻¹. For assay solution an aliquot of 1 mL was taken and then was treated as described below in the recommended procedure for the sulphanilamides determination.

2.3 General procedure of SA determination with TAR

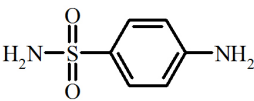
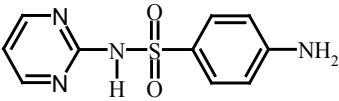
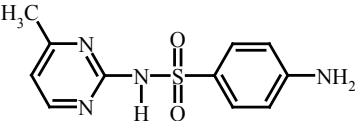
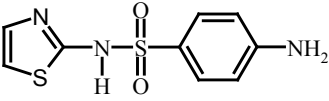
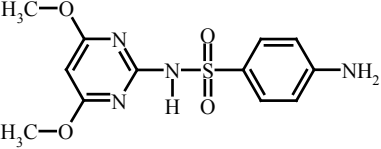
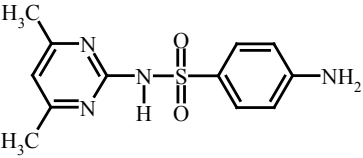
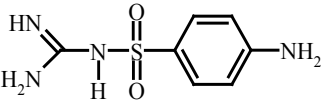
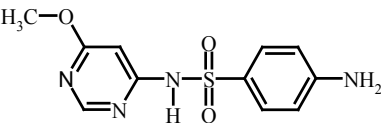
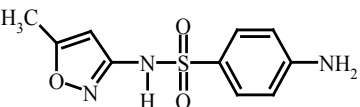
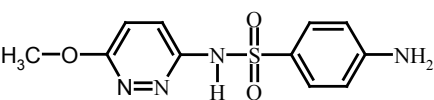
5.0 mL of 1.0 M hydrochloric acid solution was placed into a 25 mL volumetric flask. Then a sample of solution containing 1.0–30.0 µg mL⁻¹ of sulphanilamide (in final volume) was added. Next 0.5 mL of 1.25 × 10⁻² M sodium nitrite solution was added into the flask. Obtained solution was stirred and cooled on an ice bath (~ 0°C) for 10 minutes. Then 0.5 mL of 2.25 × 10⁻³ M TAR solution was added into the flask. Obtained mixture was neutralized by adding of 2.5 mL of 1.0 M sodium hydroxide solution and the pH value was adjusted to pH = 9.5 and distilled water was added to the full volume of 25 mL. Then the solution was mixed thoroughly and the absorbance measurements (at the room temperature ~20°C) were carried out against all corresponding reagents' blank solutions at 615 nm in 1.0 cm cuvettes. Sulphanilamide concentration was calculated using the methods of calibration curve and single-point standardization.

3 Results and discussion

The interaction of TAR with ten SA (Table 2), which are more frequently used in medical and veterinary practice, has been investigated.

It is established that SA do not directly interact with TAR, but their diazonium salts azocouple with azo reagent and form disazo dyes, which are characterized by a new absorbance maximum at 610 ± 5 nm (Fig. 1), the

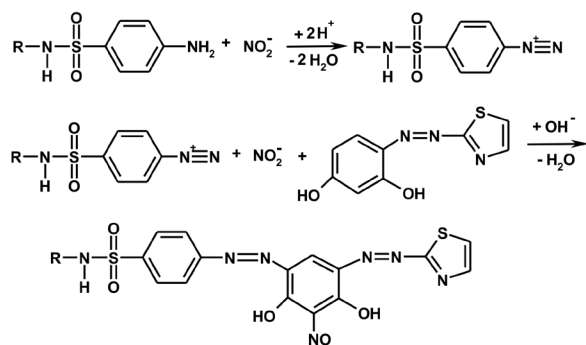
Table 2: Molecular structure of investigated sulphanilamides.

Structural formula	Name	
	Sulphanilamide (SAM) CAS 63-74-1	<i>p</i> -aminobenzene-sulphanilamide
	Sulphadiazine (SDA) CAS 68-35-9	2-(<i>p</i> -aminobenzene-sulfonamido) pyrimidine
	Sulphamerazine (SMR) CAS 127-79-7	2-(<i>p</i> -aminobenzene-sulfonamido) 4-methylpyrimidine
	Sulphathiazole (STZ), CAS 72-14-0	2-(<i>p</i> -aminobenzene-sulfonamido) 1,3-thiazol
	Sulphadimethoxine (SDM), CAS 122-11-2	6-(<i>p</i> -aminobenzene-sulfonamido) 2,4-dimethoxypyrimidine
	Sulphamethazine (SMZ), CAS 57-68-1	2-(<i>p</i> -aminobenzene-sulfonamido) 4,6-dimethylpyrimidine
	Sulphaguanidine (SGN), CAS 57-67-0	N-(<i>p</i> -aminobenzene-sulfonamido) aminoiminomethyl
	Sulphamonomethoxine (SMM), CAS 1220-83-3	4-(<i>p</i> -aminobenzene-sulfonamido) 2-methoxypyrimidine
	Sulphamethoxazole (SMX), CAS 723-46-6	3-(<i>p</i> -aminobenzene-sulfonamido) 5-methyloxazole
	Sulphamethoxy-pyridazine (SMP), CAS 000080-35-3	3-(<i>p</i> -aminobenzene-sulfonamido) 6-methoxypyridazine

absorbance value of which is directly proportional to the SA concentration.

Nitrite-ions diazotize primary aromatic amino group of sulphanilamide in acid media with the formation of

diazotized SA, which interacts with resorcinol ring of azo reagent in alkaline media. Since this ring contains two hydroxyl groups (*o*-directing substituents), which orient the azocoupling in *o*-position concordantly to any of them



Scheme 2: Scheme of SA interaction with TAR.

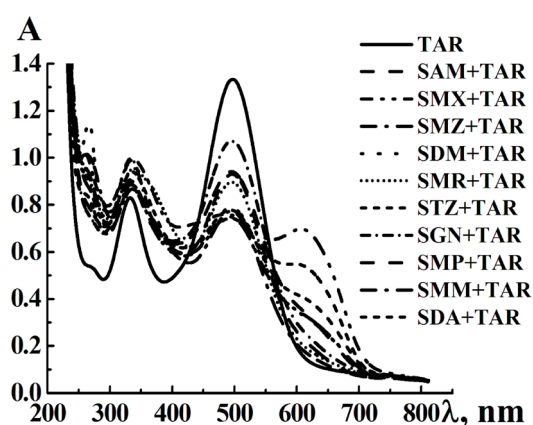


Figure 1: Absorption spectra of 4-(2-thiazolilazo)-resorcinol and solutions of the products of TAR interaction with SA diazonium salts – disazo dyes. $C_{\text{HCl}} = 1.0 \text{ M}$, $C_{\text{SA}} = 3.0 \cdot 10^{-5} \text{ M}$, $C_{\text{NaNO}_2} = 4.0 \cdot 10^{-4} \text{ M}$, $C_{\text{TAR}} = 4.5 \cdot 10^{-5} \text{ M}$, $C_{\text{Na}_2\text{B}_4\text{O}_7} = 0.01 \text{ M}$, $\text{pH } 9.5$, $l = 1 \text{ cm}$.

[44,45], the formation of only one product is possible, which is shown in the diagram (Scheme 2). In addition, as a result of action of unreacted nitrite ions, the nitroso group substitutes the hydrogen atom in the same aromatic ring in *p*-position to the both hydroxyl groups. Eventual structural formulas of the formed disazo dyes are shown in Table 3.

Conditions for SA diazotization

The conditions of SA diazotization described in the literature are rather contradictory (either hydrochloric acid in the concentration range from 0.1 M [46] to 5 M [47-49] or 10 M sulphuric acid [50-52] or a mixture of phosphoric and acetic acids [53] is used), so we have investigated the influence of various factors on the SA diazotization, such as the influence of the nature and concentration of acid for diazotization medium, diazotizing reagent, the temperature and the reaction time. As follows from the

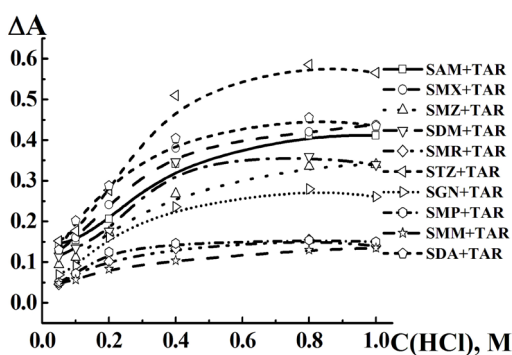
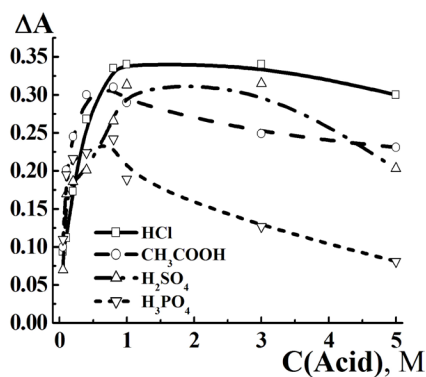


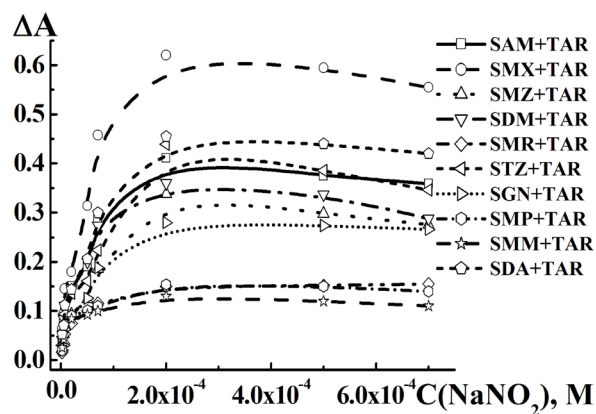
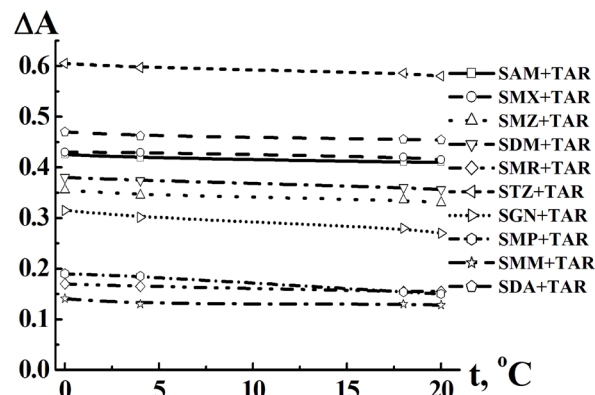
Figure 2: Effect of nature and concentration of acids (hydrochloric, sulphuric, acetic and phosphoric) on the SA interaction with TAR. $C_{\text{SA}} = 3.0 \cdot 10^{-5} \text{ M}$, $C_{\text{NaNO}_2} = 4.0 \cdot 10^{-4} \text{ M}$, $C_{\text{TAR}} = 4.5 \cdot 10^{-5} \text{ M}$, $C_{\text{Na}_2\text{B}_4\text{O}_7} = 0.01 \text{ M}$, $\text{pH } 9.5$, $l = 1 \text{ cm}$, $\lambda = 615 \text{ nm}$.

experimental data at Fig. 2, the maximum efficiency of the products of SA interaction with TAR is observed at SA diazotization in 0.5–1.0 M hydrochloric acid, using more than 10-fold excess of sodium nitrite as diazotizing reagent (Fig. 3). The use of other acids (acetic, phosphoric and sulphuric) does not allow for achieving maximum yield of diazonium salts and azocoupling product. This can be caused by the different acids strength as well as the different intermediates activity, which are formed during the SA diazotization.

It was established that the diazotization temperature affects the product yield only slightly, as both at 0°C and at room temperature (20°C), the absorbance of formed disazo dyes solutions was the same (Fig. 4). However, the diazotization time for obtaining of maximum product yield is different. At room temperature, the maximum amount of product is formed after 25–30 min, while using an ice bath the same disazo dye yield is observed already after 10 min (Fig. 5). All following researches were carried out at a room temperature while duration of diazotization was 25 min.

Table 3: Eventual structural formulas of the formed disazo dyes.

<chem>Cc1cc(C#N)nc1NS(=O)(=O)c2ccc(cc2)/N=N/c3cc(O)c(O)c([N+](=O)[O-])c3=N=N/c4cc[nH]4</chem> <u>SMX+TAR:</u>	<chem>COC1=CC=C(C=C1)N=NNS(=O)(=O)c2ccc(cc2)/N=N/c3cc(O)c(O)c([N+](=O)[O-])c3=N=N/c4cc[nH]4</chem> <u>SMP+TAR:</u>
<chem>c1cc[nH]c1NS(=O)(=O)c2ccc(cc2)/N=N/c3cc(O)c(O)c([N+](=O)[O-])c3=N=N/c4cc[nH]4</chem> <u>STZ+TAR:</u>	<chem>Nc1ccc(cc1)S(=O)(=O)N=N/c2cc(O)c(O)c([N+](=O)[O-])c2=N=N/c3cc[nH]3</chem> <u>SAM+TAR:</u>
<chem>c1cc[nH]c1NS(=O)(=O)c2ccc(cc2)/N=N/c3cc(O)c(O)c([N+](=O)[O-])c3=N=N/c4cc[nH]4</chem> <u>SDA+TAR:</u>	<chem>Nc1ccc(cc1)S(=O)(=O)N=N/c2cc(O)c(O)c([N+](=O)[O-])c2=N=N/c3cc[nH]3</chem> <u>SGN+TAR:</u>
<chem>COC1=CC=C(C=C1)N=NNS(=O)(=O)c2ccc(cc2)/N=N/c3cc(O)c(O)c([N+](=O)[O-])c3=N=N/c4cc[nH]4</chem> <u>SDM+TAR:</u>	<chem>Cc1cc(C#N)nc1NS(=O)(=O)c2ccc(cc2)/N=N/c3cc(O)c(O)c([N+](=O)[O-])c3=N=N/c4cc[nH]4</chem> <u>SMR+TAR:</u>
<chem>Cc1cc(C#N)nc1NS(=O)(=O)c2ccc(cc2)/N=N/c3cc(O)c(O)c([N+](=O)[O-])c3=N=N/c4cc[nH]4</chem> <u>SMZ+TAR:</u>	<chem>COC1=CC=C(C=C1)N=NNS(=O)(=O)c2ccc(cc2)/N=N/c3cc(O)c(O)c([N+](=O)[O-])c3=N=N/c4cc[nH]4</chem> <u>SMM+TAR:</u>

**Figure 3:** Effect of sodium nitrite concentration during SA diazotization on the SA interaction with TAR. $C_{\text{HCl}} = 1.0 \text{ M}$, $C_{\text{SA}} = 3.0 \cdot 10^{-5} \text{ M}$, $C_{\text{TAR}} = 4.5 \cdot 10^{-5} \text{ M}$, $C_{\text{Na}_2\text{B}_4\text{O}_7} = 0.01 \text{ M}$, pH 9.5, $l = 1 \text{ cm}$, $\lambda = 615 \text{ nm}$.**Figure 4:** Effect of temperature during SA diazotization on disazo dyes formation. $C_{\text{HCl}} = 1.0 \text{ M}$, $C_{\text{SA}} = 3.0 \cdot 10^{-5} \text{ M}$, $C_{\text{NaNO}_2} = 4.0 \cdot 10^{-4} \text{ M}$, $C_{\text{TAR}} = 4.5 \cdot 10^{-5} \text{ M}$, $C_{\text{Na}_2\text{B}_4\text{O}_7} = 0.01 \text{ M}$, pH 9.5, $l = 1 \text{ cm}$, $\lambda = 615 \text{ nm}$.

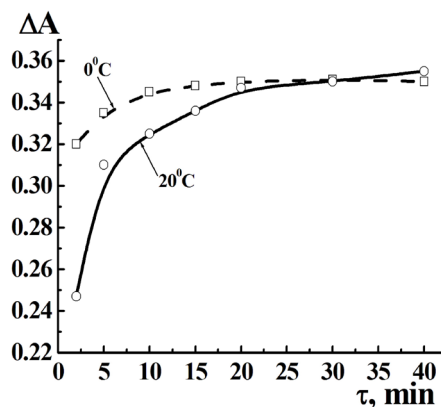


Figure 5: Effect of time of sulphanilamide diazotization at different temperatures on the disazo dye yield. $C_{\text{HCl}} = 1.0 \text{ M}$, $C_{\text{SA}} = 3.0 \cdot 10^{-5} \text{ M}$, $C_{\text{NaNO}_2} = 4.0 \cdot 10^{-4} \text{ M}$, $C_{\text{TAR}} = 4.5 \cdot 10^{-5} \text{ M}$, $C_{\text{Na}_2\text{B}_4\text{O}_7} = 0.01 \text{ M}$, pH 9.5, $l = 1 \text{ cm}$, $\lambda = 615 \text{ nm}$.

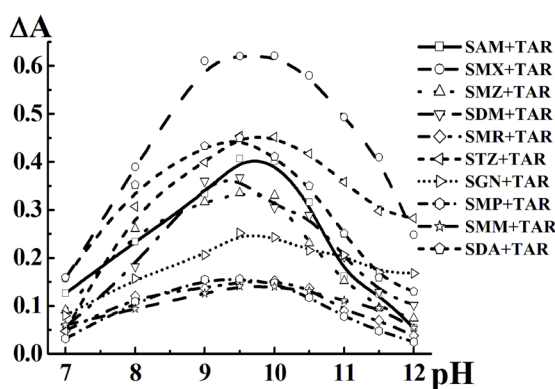


Figure 6: Effect of medium acidity on diazotized sulphanilamides azocoupling with TAR. $C_{\text{HCl}} = 1.0 \text{ M}$, $C_{\text{SA}} = 3.0 \cdot 10^{-5} \text{ M}$, $C_{\text{NaNO}_2} = 4.0 \cdot 10^{-4} \text{ M}$, $C_{\text{TAR}} = 4.5 \cdot 10^{-5} \text{ M}$, $C_{\text{Na}_2\text{B}_4\text{O}_7} = 0.01 \text{ M}$, $l = 1 \text{ cm}$, $\lambda = 615 \text{ nm}$.

Azocoupling conditions of TAR with SA diazonium salts

To establish optimal conditions for SA diazonium salts interaction with TAR, the effect of acidity on the azocoupling reaction, required reagent excess, nature and concentration of anions for selection of the buffer mixture were investigated.

The azocoupling reaction between diazonium salt and azo reagent occurs in an alkaline medium at pH 9–10 (Fig. 6). In this acidity range TAR exists as HR^- ions containing resorcinol ring with single-dissociated OH group in its structure. HR^- form of TAR promotes electrophilic substitution of one hydrogen atom of resorcinol ring with the SA diazonium salt (weak electrophile). Obtained results of investigation are well

correlated with the literature data [44,45], therefore in following experiments we carried out azocoupling reaction at pH 9.5.

To improve the technique's rapidity as well as stabilization of the reaction conditions, the influence of the nature and concentration of various salts anions on the azocoupling reaction for the selection of buffer solution has been investigated. According to the experimental results (Fig. 7), the presence of nitrate, acetate, carbonate, phosphate, sulphate anions in the reaction mixture as well as ammonium, calcium, and magnesium cations adversely affects the yield of the final product, whereas the use of low concentrations of ammonia buffer solution and potassium bromide does not affect the analytical signal. The use of universal buffer mixture (UBM), pyrophosphate, tetraborate anions slightly increases of analytical signal. Therefore, sodium tetraborate with concentration of 0.04 M in the final volume has been used as a buffer solution.

It is known that certain excess amount of reagent promotes a shift of reaction equilibrium toward the formation of reaction products that makes it possible to get the maximum yield of the colored products. We found that for maximum yield of disazo dye 1.5-fold azo reagent excess is required, while the increase of TAR concentrations does not affect the analytical signal (Fig. 8). According to the both mole ratio method (Fig. 8) and the method of continuous variations (Fig. 9) the stoichiometric SA:TAR ratios is 1:1.

It was established that the order of reagents addition is important and maximum yield of colored products is observed when reagents are added as follows: solution of hydrochloric acid, SA solution, sodium nitrite solution, the next SA diazotization is carried out, then TAR and buffer solutions are added, then the pH of mixture is adjusted to required value.

An important feature of the method of the substance determination is stability of analytical form, which induces analytical signal. As follows from obtained data (Fig. 10), absorbance value of disazo dyes of all examined SA did not change for 1–2 hours, what is sufficient for assay.

Research of product of SA diazonium salt azocoupling with TAR

Diazonium salts of SA are obtained in acidic medium under a 10-fold excess of sodium nitrite. Under these conditions maximum yield of diazonium salts and subsequently the products of their interaction with azo reagent is ensured. We have compared the absorption spectra (Fig. 11) of the products of SA diazonium salts interaction with TAR at the example of SMZ both in the case of sodium nitrite

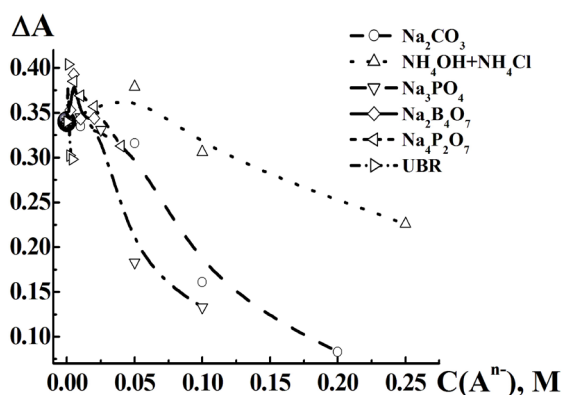
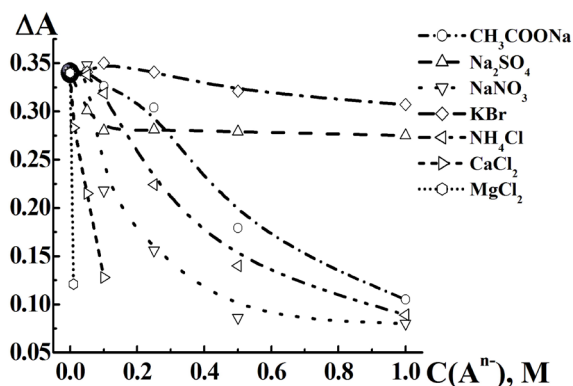


Figure 7: Influence of nature and concentration of salts anions (acetate, sulphate, bromide, nitrate, phosphate, pyrophosphate, carbonate, tetraborate); buffer solutions (ammonia, UBM); cations (ammonium, calcium) on the SA interaction with TAR. $C_{\text{HCl}} = 1.0 \text{ M}$, $C_{\text{SA}} = 3.0 \cdot 10^{-5} \text{ M}$, $C_{\text{NaNO}_2} = 4.0 \cdot 10^{-4} \text{ M}$, $C_{\text{TAR}} = 4.5 \cdot 10^{-5} \text{ M}$, pH 9.5, $l = 1 \text{ cm}$, $\lambda = 615 \text{ nm}$.

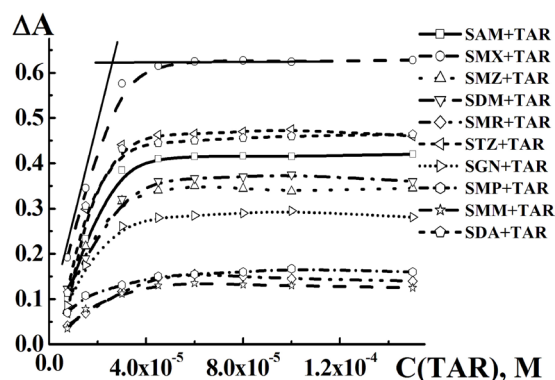


Figure 8: The mole ratio curve for disazo dyes of diazotized sulphanilamides and TAR. $C_{\text{HCl}} = 1.0 \text{ M}$, $C_{\text{SA}} = 3.0 \cdot 10^{-5} \text{ M}$, $C_{\text{NaNO}_2} = 4.0 \cdot 10^{-4} \text{ M}$, $C_{\text{Na}_2\text{B}_4\text{O}_7} = 0.01 \text{ M}$, pH 9.5, $l = 1 \text{ cm}$, $\lambda = 615 \text{ nm}$.

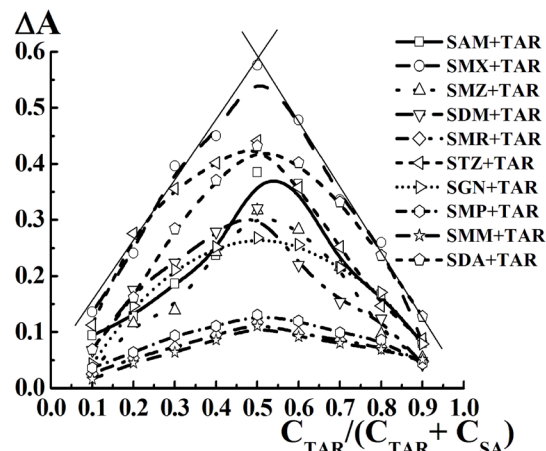


Figure 9: The continuous variations curve for disazo dyes of diazotized sulphanilamides and TAR. $C_{\text{HCl}} = 1.0 \text{ M}$, $C_{\text{NaNO}_2} = 4.0 \cdot 10^{-4} \text{ M}$, $C_{\text{SA+TAR}} = 6.0 \cdot 10^{-5} \text{ M}$, $C_{\text{Na}_2\text{B}_4\text{O}_7} = 0.01 \text{ M}$, pH 9.5, $l = 1 \text{ cm}$, $\lambda = 615 \text{ nm}$.

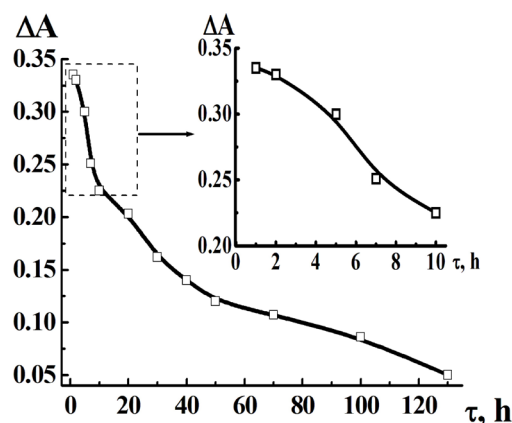


Figure 10: Effect of the keeping time on the absorbance of disazo dye solution. $C_{\text{HCl}} = 1.0 \text{ M}$, $C_{\text{SMZ}} = 3.0 \cdot 10^{-5} \text{ M}$, $C_{\text{NaNO}_2} = 4.0 \cdot 10^{-4} \text{ M}$, $C_{\text{TAR}} = 4.5 \cdot 10^{-5} \text{ M}$, $C_{\text{Na}_2\text{B}_4\text{O}_7} = 0.01 \text{ M}$, pH 9.5, $l = 1 \text{ cm}$, $\lambda = 615 \text{ nm}$.

residue presence as well as its removal from the reaction medium under the action of urea.

According to the experimental results (Fig. 11), nitrite-ions, which are present in reaction medium affect the azo reagent, because the new absorption maximum at 340 nm on the reagent spectrum appears and a decrease in absorbance of TAR maximum at 500 nm is observed. The presence of urea in the reaction medium does not affect the absorption spectrum of azo reagent and its 50-fold excess to sodium nitrite in solution before adding of azo reagent completely destroys nitrite-ions, as evidenced by the same character of reagent spectrum both in case of the presence of unreacted sodium nitrite and its absence.

Also, we used the method of voltammetry, in particular oscillographic polarography, which gives the possibility to explore the reduction nature of the obtained compounds on the mercury dropping electrode. These investigations can help to confirm our hypothesis concerning mechanism of SA diazonium salts interaction with 4-(2-thiazolilazo)-resorcinol.

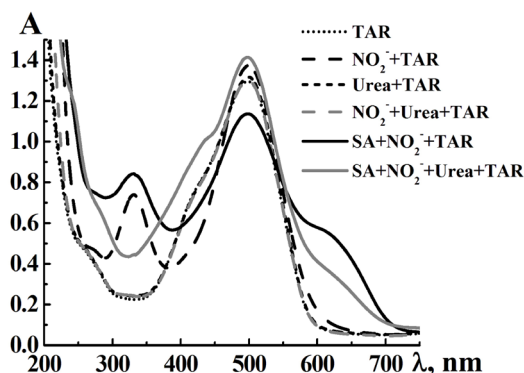


Figure 11: Absorption spectra of solutions of TAR as well as products of its interaction with SMT diazonium salt, which were obtained without elimination and after the elimination of unreacted sodium nitrite by the urea. $C_{\text{HCl}} = 1.0 \text{ M}$, $C_{\text{SA}} = 3.0 \cdot 10^{-5} \text{ M}$, $C_{\text{NaNO}_2} = 4.0 \cdot 10^{-4} \text{ M}$, $C_{\text{TAR}} = 4.5 \cdot 10^{-5} \text{ M}$, $C_{\text{Na}_2\text{B}_4\text{O}_7} = 0.01 \text{ M}$, pH 9.5, $l = 1 \text{ cm}$.

In voltamperograms of the process of the TAR reduction (Fig. 12a) as well as azo reagent reduction in the presence of urea (Fig. 12b) a peak of azo group reduction at potential -0.70 V is observed. In the presence of nitrite-ions on polarogram of TAR reduction two peaks are observed: the peak at -0.72 V is characteristic to azo group reduction and the peak at -0.32 V is corresponding to reduction of nitroso group, introduced in the azo reagent benzene ring under the sodium nitrite action (Fig. 12c). After the nitrite destruction by the urea the peak at -0.32 V on the polarogram of azo reagent reduction was absent (Fig. 12d).

There are two peaks, at -0.71 V and at -0.85 V on the polarograms of reduction of TAR interaction products with diazotized SA at the potentials corresponding to azo group reduction. They indicate the reduction of two azo groups: the first one is azo group of TAR molecules, and the other is new azo group formed as a result of the SA diazonium salt azocoupling with resorcinol group of azo compound. At -0.32 V, the peak of reduction of the nitroso group, which under the nitrite ions excess is included into the product molecule (Fig. 12e) is observed. Product, which was formed in the azocoupling reaction after the destruction of nitrite ions excess, does not contain in its structure nitroso group, as evidenced by the absence of the peak at -0.42 V, while in polarogram of product reduction two peaks of two azo groups are observed (Fig. 12f).

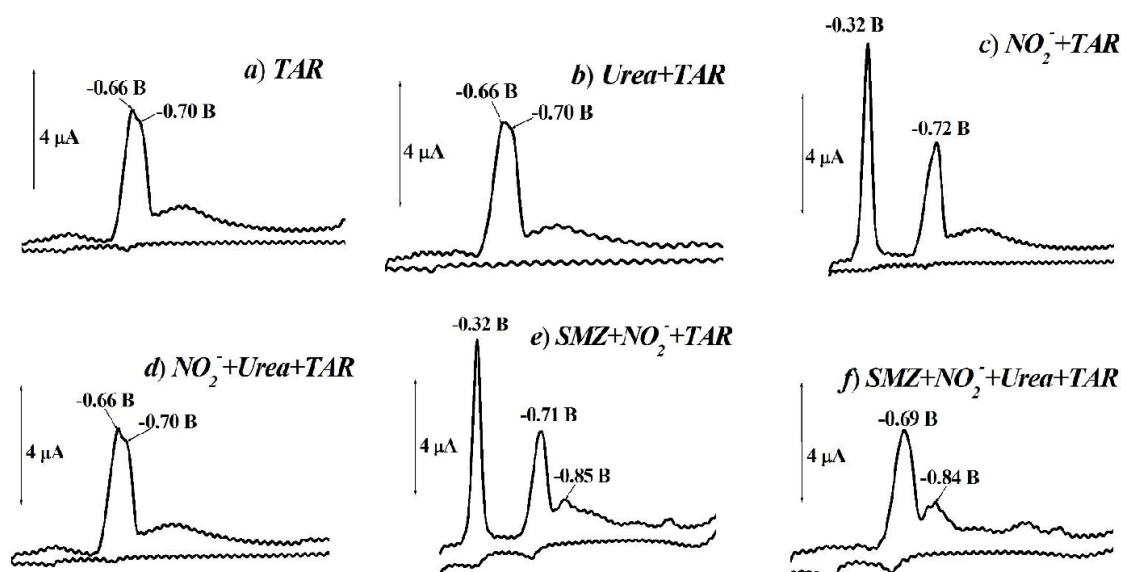


Figure 12: Polarograms of the reduction process of the TAR (a), TAR in the presence of urea (b), TAR in the presence of sodium nitrite (c), TAR after the destruction of unreacted sodium nitrite by urea (d), products of the SMZ diazonium salt interaction with TAR without elimination of sodium nitrite excess (e), product of the SMZ diazonium salt interaction with TAR after the destruction of sodium nitrite excess by the urea (f). $C_{\text{HCl}} = 1.0 \text{ M}$, $C_{\text{SA}} = 3.0 \cdot 10^{-5} \text{ M}$, $C_{\text{NaNO}_2} = 4.0 \cdot 10^{-4} \text{ M}$, $C_{\text{TAR}} = 4.5 \cdot 10^{-5} \text{ M}$, $C_{\text{Na}_2\text{B}_4\text{O}_7} = 0.01 \text{ M}$, pH 9.5.

Table 4: Spectroscopic characteristics of the formed disazo dyes and validation results of sulphanilamides spectrophotometric determination with TAR. $C_{\text{HCl}} = 1.0 \text{ M}$, $C_{\text{NaNO}_2} = 1.2 \times 10^{-3} \text{ M}$, $C_{\text{TAR}} = 1.8 \times 10^{-4} \text{ M}$, $C_{\text{Na}_2\text{B}_4\text{O}_7} = 0.01 \text{ M}$, $\text{pH} = 9.5$, $l = 1 \text{ cm}$, $n = 5$, $P = 0.95$.

Parameters/Characteristics	SAM	SMX	SMZ	SDM	SMR	STZ	SGN	SMP	SMM	SDA
$\Delta\lambda_{\text{max}}$, nm	615									
Stability, hours	1									
Optimum photometric linear range, $\mu\text{g mL}^{-1}$	0.5–10.0	1.0–12.0	1.0–24.0	1.0–24.0	3.0–30.0	0.8–18.0	2.0–30.0	2.5–30.0	3.0–30.0	0.8–18.0
Limit of Detection, $\mu\text{g mL}^{-1}$	0.17	0.33	0.38	0.33	0.88	0.25	0.75	0.83	0.94	0.28
Limit of Quantification, $\mu\text{g mL}^{-1}$	0.55	1.08	1.27	1.08	2.91	0.84	2.48	2.75	3.09	0.83
Molar absorptivity, $\epsilon_{615} \times 10^{-4}, \text{M}^{-1} \text{cm}^{-1}$	0.7	2.2	1.1	1.2	0.6	1.7	0.7	0.8	0.6	1.3
Regression equation (A) ^a Slope (b)	0.090	0.083	0.039	0.046	0.017	0.059	0.030	0.018	0.016	0.054
Intercept (a)	0.012	0.014	0.011	0.010	0.012	0.015	0.017	0.008	0.001	0.016
Correlation coefficient (R)	0.9997	0.9998	0.9999	0.9998	0.9996	0.9996	0.9996	0.9998	0.9997	0.9997

^aA = bC + a, where C is the concentration of sulphanilamide in $\mu\text{g mL}^{-1}$.

As follows from Fig. 12 the obtained results are in accordance with spectrophotometric studies and confirm the proposed scheme of 4-(2-thiazolilazo)-resorcinol interaction with SA diazonium salts.

Validation and spectroscopic characteristics of the developed method

On the basis of the optimal conditions of SA interaction with TAR the new spectrophotometric technique of SA determination has been developed. Validation and spectroscopic characteristics of SA determination are presented in Table 4.

As follows from the Table 4 and Scheme 3 the absorbance value of colored products of SA interaction with TAR depends on the nature of the substituent in the sulphanilamide molecule, that in its turn appears on the validation characteristics of each SA determination methods. Five-membered heterocycles with two different heteroatoms in the molecules of SA (STZ, SMX) show the greatest effect on the analytical signal. SA whose molecules contain the six-membered heterocycles with two identical heteroatoms and two substituents can be determined with higher sensitivity than SA, containing the same heterocycles with one substituent. The method of non-substituted sulphanilamide streptocide determination has the lowest sensitivities. All these effects are related to the distribution of electron density in the molecules of both the SA and TAR, as well as in the products of their interaction, and also to the availability of mesomeric and inductive effects of substituents [44,45]. The results of investigation correlate with the results obtained in the

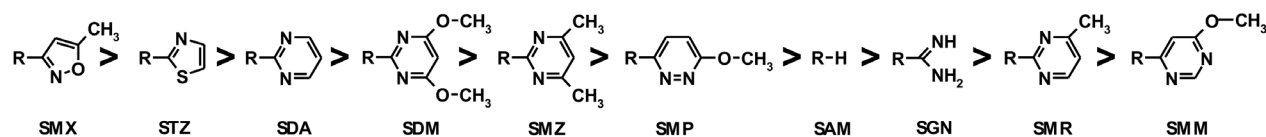
study of the interaction of SA diazonium salts with another heterocyclic azo reagent 4-(2-pyridylazo) resorcinol [39,40] and acid monoazo dye Tropaeolin O [37,38].

Analysis of pharmaceutical preparations for the SA content with TAR

Multi- and one-component sulphanilamides drugs are widely used both in medical and in veterinary practice. Assortment of medicaments for animals is much wider and more diverse due to combination of SA with various medicinal substances with different chemical nature. Developed methods of spectrophotometric SA determination with TAR have been successfully used to establish the SA content in one- and two-component drugs of various dosage forms namely: tablets, solution, and ointment.

Previously, the effect of bioactive substances and common pharmaceutical excipients on the procedure of SA determination (using SMZ) by means of TAR was investigated. Moreover selectivity of the developed method was tested for mixtures containing excipients in amounts greatly exceeding their possible content in pharmaceutical preparations. Mutability of absorbance value for formed disazo dye SA-TAR in the range $\pm 5\%$ was elected as the selectivity criterion of SA determination. The research results are presented in Table 5.

The obtained results show that the excipients and bioactive substances, which are present in preparations, do not interfere with the SA determination by the developed technique with TAR. The results of SA determination in veterinary medicines are given in Table. 6.



Scheme 3: Series of sensitivity decrease for sulphanilamides determination at azocoupling reaction with TAR.

Table 5: Effect of Excipients on the SA assays by TAR. $C_{HCl} = 1.0$ M, $C_{SA} = 3.0 \times 10^{-5}$ M, $C_{NaNO_2} = 4.0 \times 10^{-4}$ M, $C_{TAR} = 4.5 \times 10^{-5}$ M, $C_{Na_2B_4O_7} = 0.01$ M, pH 9.5, $l = 1$ cm, $P = 0.95$, $n = 3$.

Excipient (Exp)	m(SA) : m(Exp)*	m(SA) : m(Exp)**	% Recovery of SA, $\bar{x} \pm S \cdot t_{\alpha} / \sqrt{n}$
Trimethoprim	1:0.2	1:10	97.4±1.4
Enrofloxacin	1:0.25	1:50	98.3±1.6
Gentamicin	1:0.4	1:10	96.4±1.9
Starch	1:0.1	1:2	100.4±1.4
Cellulose	1:0.1	1:10	99.1±1.9
Gelatine	1:0.1	1:10	95.5±1.6
Talc	1:0.03	1:2	99.2±2.0
Aerosil	1:0.1	1:2	96.9±1.8
Calcium stearate	1:0.01	1:10	103.6±2.1
Propylene glycol	1:0.025	1:2	99.5±1.6
Vaseline	1:9	1:25	96.4±1.8
Benzyl alcohol	1:0.01	1:0.5	95.4±2.0
Polysorbate 80	1:0.01	1:10	95.2±2.0
Ethyl benzoate	1:0.01	1:1	96.0±1.7
Methylparaben	1:0.005	1:1	98.6±1.6
Propylparaben	1:0.001	1:10	97.1±1.8

*- mass ratios of SA and excipients, which are present in tested drugs [54]

** - maxima mass ratios of SA and excipients, which were examined

As can be seen from the Table 6, the SA content in medicines obtained due to the developed technique using TAR correlates rather well with the content of the SA specified by the manufacturer as well as with the results obtained according to the official methods of nitritometry. However, the developed method is much simpler and, unlike the official methods, it does not require extraction at the analysis of combined drugs; moreover, it is characterized by high reproducibility and rapidity. By sensitivity, this reaction does not differ from the other most sensitive spectrophotometric methods of SA determination. The value of S_r does not exceed common values of spectrophotometry errors.

Also, the validation of the developed method of sulphamethazine determination in combination

Table 6: Determination of SA in pharmaceuticals. $C_{HCl} = 1.0$ M, $C_{NaNO_2} = 4.0 \times 10^{-4}$ M, $C_{TAR} = 4.5 \times 10^{-5}$ M, $C_{Na_2B_4O_7} = 0.01$ M, pH 9.5, $l = 1$ cm, $P = 0.95$, $n = 5$.

Determined SA (regulated content in preparation)	Amount of found drug $\bar{x} \pm \Delta x$ and relative standard deviation (S)	
	Pharmaceutical method (nitritometry) [36]	Spectrophotometric method with TAR
„Trymeratynvet” tablets, OJSC “VetsynteZ”, Kharkiv (trimethoprim (80 mg/tab), excipients – starch, talc, calcium stearate)		
Sulphamethazine (400 ± 40 mg/tab)	397 ± 10 (0.025)	399 ± 9 (0.021)
„Streptocid ointment 10%”, “Brovapharma LTD”, Brovary (excipients – vaseline)		
Sulphanilamide (100 ± 10 mg g ⁻¹)	100 ± 2 (0.020)	100 ± 1 (0.012)
„Floxi-C” solution, LLC “Vetmedpreparaty”, Kyiv (enrofloxacin (50 mg mL ⁻¹), excipients – propylene glycol, water)		
Sulphadimethoxine (200 ± 20 mg mL ⁻¹)	201 ± 5 (0.025)	202 ± 7 (0.030)
„Gentazimvet” powder, OJSC “VetsynteZ”, Kharkiv (trimethoprim (20 mg g ⁻¹), gentamicin (40 mg g ⁻¹), excipients – starch, talc, calcium stearate)		
Sulphamethazine (100 ± 10 mg g ⁻¹)	103 ± 2 (0.019)	101 ± 3 (0.021)
„Sulphadimethoxine” tablets, PJSC SIC „Borshchahivskiy CPP”, Kyiv (excipients – starch, calcium stearate, gelatine, aerosil)		
Sulphadimethoxine (500 ± 50 mg/tab)	501 ± 8 (0.14)	499 ± 8 (0.013)
„Trisulfon” suspension «KRKA» Slovenia (trimethoprim (80 mg mL ⁻¹), excipients – cellulose, polysorbate 80, ethyl benzoate, methylparaben, propylparaben)		
Sulphamethoxazole (400 ± 40)	425 ± 16 (0.037)	410 ± 18 (0.038)

veterinary drug “Vettrymerazyn” solution, which also contains bioactive substances trimethoprim and excipients such as propylene glycol, benzyl alcohol and water, has been carried out. The following characteristics – linearity, accuracy, and precision in the range of 50–150 % from the nominal content of SMZ in preparation, and intermediate precision have been validated according to [32,33,35,55]. The results are presented in Tables 7 and 8.

Table 7: Criteria of linearity, accuracy, and precision of SMZ determination in preparation “Vettrymerazyn” solution.

Name of solution	$C_p, \mu\text{g mL}^{-1}$	$X_i = \frac{C_i}{C_{St}} \cdot 100\%$	A_i	$Y_i = \frac{A_i}{A_{St}} \cdot 100\%$	$D_i = \frac{\textit{found}}{\textit{added}} \cdot 100\%$
Reference solution	8.012	100.00	0.331	100.00	100.00
No1 (50.0%)	4.008	50.02	0.172	50.45	100.86
No 2 (50.0%)	4.008	50.02	0.175	49.55	99.06
No 3 (50.0%)	4.008	50.02	0.175	50.24	100.43
No 4 (100.0%)	8.016	100.05	0.333	101.16	101.11
No 5 (100.0%)	8.016	100.05	0.335	100.22	100.17
No 6 (100.0%)	8.016	100.05	0.336	101.09	101.04
No 7 (150.0%)	12.024	150.07	0.489	150.91	100.56
No 8 (150.0%)	12.024	150.07	0.490	149.27	99.47
No 9 (150.0%)	12.024	150.07	0.492	151.44	100.91
\overline{D}					100.40
Criteria of linearity					
Parameters	Value		Criteria		Conclusion
b	1.004		–		The first criterion is met
S_b	0.006		–		
a	0.024		1) $\leq 1.273 $ 2) $\leq 2.048 $		
S_a	0.672		–		
r	0.9998		$\geq 0.9803 $		The criterion is met
The criteria of accuracy and precision					
The relative standard deviation $RSD_o = 0.718\%$					
Critical values of the one-sided confidence interval, $\Delta x = 1.34$			$\Delta x \leq 3.2\%$	Satisfies the requirements	
Criterion of the systematic error insignificance, $\delta = 0.40\%$			1. $\delta \leq 0.447\%$ 2. $\delta \leq 1.024\%$	Satisfies the requirements (the first criterion)	
The overall conclusion on methods					Is correct

Calculated validation parameters of SMZ determination methods in preparation „Vettrymerazyn“ using heterocyclic azo reagent TAR satisfied the corresponding criteria, which allowed us to suggest that the developed method is suitable for the quality control of this drug in terms of „Assay“.

4 Conclusions

For the first time the formation of disazo dyes by the interaction of diazotized sulphanilamides with heterocyclic azo reagent 4-(2-thiazolylazo)-resorcinol has been established. Optimum conditions for ten sulphanilamides diazotization as well as following azocoupling with TAR have been investigated. It is established that the

components ratio in the disazo dyes is SA:TAR = 1:1. It has been demonstrated that azocoupling occurs due to the cresol group of second component (TAR), which has concordantly oriented substitutes. Spectroscopic and validation characteristics of sulphanilamides determination with TAR have been established. The correlation between the SA structure and sensitivity of their determination with TAR has been detected. The series of sensitivity of ten SA determinations with azo dye has been established. The effective molar absorptivity ε_{615} is $\sim 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. The proposed method is found to be simple, rapid, sensitive, selective, and economical. It allows determining wide range of SA concentrations ($0.5\text{--}30.0 \mu\text{g mL}^{-1}$ depending on the detectable sulphanilamide) and competes with most of the spectrophotometric methods available in literature.

Table 8: Test results of intermediate precision of SMZ quantitative determination in preparation „Vettrymerazyn.“ $C_{\text{HCl}} = 1.0 \text{ M}$; $C_{\text{NaNO}_2} = 4 \times 10^{-4} \text{ M}$; $C_{\text{TAR}} = 4.5 \times 10^{-5} \text{ M}$; $C_{\text{Na}_2\text{B}_4\text{O}_7} = 0.01 \text{ M}$; $\text{pH} = 9.5$; $l = 1 \text{ cm}$.

Analysis No	m, mg/5 ml	
	1 st day (experiment 1)	2 nd day (experiment 2)
1	195.3	196.5
2	188.1	187.3
3	199.6	191.4
4	204.7	205.7
5	204.5	203.9
average (\bar{m})	198.4	197.0
combined average (\bar{m}_{intra})	197.7	
S_m	7.0	7.9
S_{intra} [56,57]	7.4	
Δ_{intra} [56,57]	6.1	

The method is advantageous over many spectrophotometric methods with special reference to stability and sensitivity. The validation parameters clearly indicate the reproducibility ($S_r \leq 0,038$) and accuracy of the method. The elaborated method has been approved during analysis of single-component and combined commercial pharmaceuticals in the presence of different classes of biologically active substances. Obtained data were well correlated to those obtained using the official methods of nitritometry for each of the studied dosage forms (tablets, ointment, solution, powder, suspension). Therefore the recommended procedure is well suited for the assay of drugs to assure high standard of quality control.

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