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ARTICLES

Spectrophotometric and Fluorimetric Determination of Amino Acids by Their Reaction with *o*-Phthalic Aldehyde in the Presence of Sulfite and Cyanide Ions

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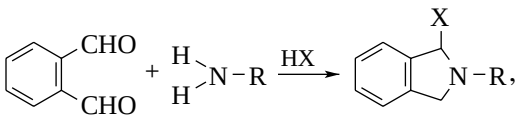
Abstract—Optimum conditions were selected for the determination of amino acids by their reaction with *o*-phthalic aldehyde in the presence of sodium sulfite and potassium cyanide using glycine as an example. It was shown that the analytical characteristics of the proposed procedure are highly competitive with those of a widely employed procedure using 2-mercaptoethanol and that the stability of the analytical form (substituted isoindols) was much higher.

The reactions of *o*-phthalic aldehyde (OPA) with amino compounds in the presence of nucleophilic agents have been extensively used for the sensitive electrochemical, spectrophotometric, and fluorimetric determination of primary amines and amino acids. Since the first reports on the formation of highly fluorescent products in this reaction [1], extensive studies of the reaction kinetics and mechanism have been carried out, the reaction products were detected, and their spectrophotometric characteristics were determined [2–4]. The following reaction mechanism is well established for the reaction of *o*-phthalic aldehyde with amino acids:



where R is the amino acid residue, HX is the nucleophilic agent, and I is the substituted isoindol. Alkylmercaptans, thioalcohols and organic thioacids, as well as sulfite and cyanide ions can act as nucleophilic agents. Until now, 2-mercaptoethanol (2ME) was the most commonly used nucleophilic agent. 2-Hydroxyethylsulfanyl-N-substituted isoindols formed in the reaction exhibit high fluorescence efficiency; however, the low stability of the products deteriorates the analytical characteristics of the procedure for the determination of amino acids, especially when it is combined with HPLC [5]. At present, the search for reagents for the highly sensitive determination of amines and amino acids is made in two directions: (1) the optimization of conditions for the reaction with OPA, including the selection of nucleophilic agents that will provide the most stable and highly fluorescent or light-absorbing products [6] and (2) the synthesis of OPA analogs containing aldehyde or keto groups in *ortho*-positions [7, 8].

This work is dedicated to the search for the optimum conditions for the reaction between amino acids and *o*-phthalic aldehyde, the determination of the analytical characteristics of the procedure using sulfite and cyanide ions as nucleophilic agents, and the comparison of the proposed procedure with a standard one using 2ME as a nucleophilic agent. The studies were carried out with aminoacetic acid, which gives the least stable products among those produced by the monoamino acids [4].

EXPERIMENTAL

Absorption spectra and absorbances were measured on a Specord UV-VIS spectrophotometer (Karl Zeiss, FRG); fluorescence spectra were recorded on a Flyuorat-Panorama spectrofluorimeter (Lyumeks, St. Petersburg) (Fig. 1); and kinetic curves of the formation and decomposition of fluorescent products were obtained with a Flyuorat-2A fluorimeter (Lyumeks, St. Petersburg). The light filters to the fluorimeter were selected with regard to the absorption and fluorescence spectra of the studied substance: for fluorescence excitation, a filter with a band width of 300–340 nm and a transmission maximum of 316 nm (filter no. 3) and, for emission, a filter with a band width of 370–420 nm and a transmission maximum of 395 nm (filter no. 13). The aminoacetic acid, NH_4Cl, NaOH, CH_3COOH, and KCN were of reagent grade; the Na_2SO_3 was of analytical grade; the methanol (Merck, FRG) was of chromatography grade. A borate buffer solution (0.1 M) of pH 9.18 was prepared from volumetric standards (RIAP, Moscow). The water was purified with a MilliQ system for water preparation (Millipore, USA). The mercaptoethanol for synthesis (Serva, USA) was purified by distillation in a vacuum produced by a water-jet pump. The *o*-Phthalic aldehyde for synthesis (Merck, FRG) was purified by sublimation in vacuum. The

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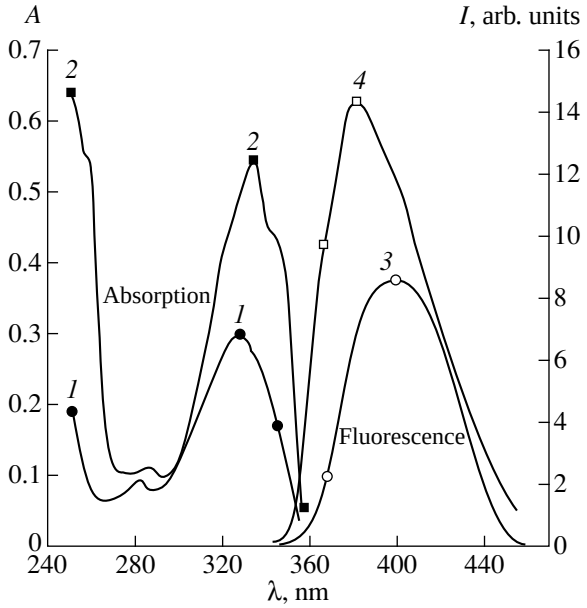

Fig. 1. (*1*, *2*) Absorption and (*3*, *4*) fluorescence spectra of the products of the reaction between 5×10^{-5} M glycine and 2×10^{-4} M OPA in the presence of (*1*, *3*) 2×10^{-2} M Na_2SO_3 and (*2*, *4*) 1×10^{-3} M KCN within (*3*) 60 and (*1*, *2*, and *4*) 15 min after mixing the reagent solutions.

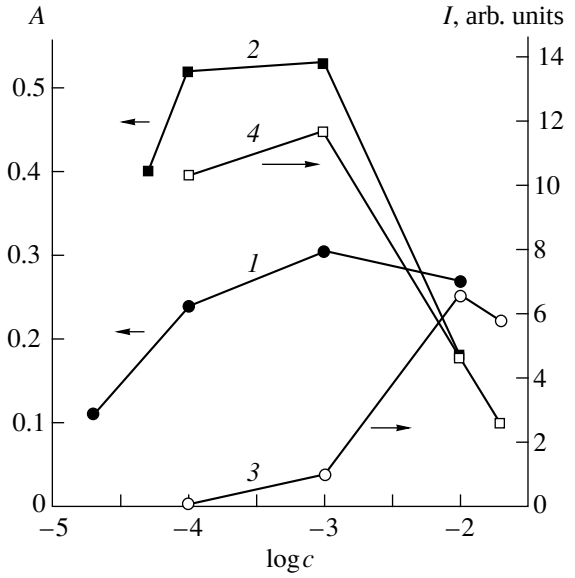

Fig. 2. Effect of the concentrations of nucleophilic agents (*c*, M) on (*1*, *2*) absorbances and (*3*, *4*) fluorescence intensities of the products of the reaction between OPA and glycine measured at absorption and fluorescence maxima in the presence of (*1*, *3*) Na_2SO_3 and (*2*, *4*) KCN within (*3*) 60 and (*1*, *2*, and *4*) 15 min after mixing the reagent solutions.

purity of the latter two substances was monitored by HPLC with atomic-emission detection. An HP6890 gas chromatograph (Hewlett–Packard Company) with an autosampler, an HP2350A detector, an HP-1 capillary column 25 m in length and 0.32 mm in the inner diameter with methyl silicon was used. The stream split ratio was 1 : 100; the carrier gas (helium)flow rate was 10 mL/min; column temperature was linearly programmed from 40 to 200°C. Carbon, sulfur, and nitrogen were detected by emission bands at 193, 181, and 174 nm, respectively. The choice of standard conditions for detection in discharge was governed by the recommendations of the Hewlett–Packard Company. We managed to obtain OPA and 2ME of no less than 99% purity with respect to carbon and 2ME of no less than 98.5% purity with respect to sulfur. Nitrogen-containing impurities were detected in none of the substances.

Preparation of solutions. The calculated amount of OPA was dissolved in 5 mL of methanol in a volumetric flask and diluted to the mark with a borate buffer solution. 2ME was added with a microsyringe to 5 mL of methanol, the solution was stirred, placed in a volumetric flask, and diluted to the mark with a borate buffer solution. Calculated samples of Na_2SO_3 and KCN were placed in volumetric flasks, dissolved in a borate buffer solution, and diluted to the mark. A sample of glycine was dissolved in water in a volumetric flask and diluted to the mark to obtain a 1×10^{-3} M solution. The solutions obtained were kept for no longer than 7 days. Standard solutions of glycine were prepared by diluting the stock solutions to the required concentration. For measurements and the construction of calibration graphs, the calculated volumes of the buffer solution, solutions of OPA, nucleophilic agents, and glycine were placed in test tubes to the total volume of 10 mL, and the mixture was stirred. All reactions were performed at 22 ± 2°C.

RESULTS AND DISCUSSION

Substituted isoindols formed in reactions of amino acids with OPA are rather reactive substances [9]. They are readily oxidized with dissolved atmospheric oxygen and react with excess OPA and nucleophilic agents.

Table 1. Optimum conditions for the determination of aminoacetic acid by its reaction with OPA

Nucleophilic agent	Optimum concentration, M		Optimum reaction time, min	
	spectrophotometry	fluorimetry	spectrophotometry	fluorimetry
KCN	$(1–10) \times 10^{-4}$	$(1–10) \times 10^{-4}$	15	15
Na_2SO_3	1×10^{-3}	1×10^{-2}	15	60

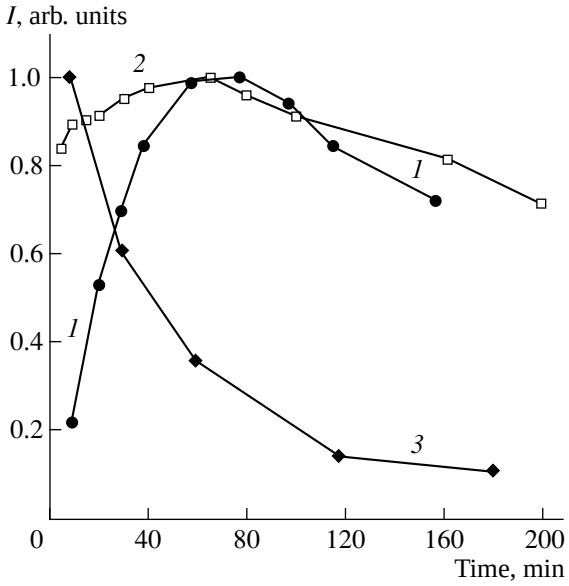

Fig. 3. Fluorescence kinetic curves for the formation and decomposition of the products of the reaction between OPA and glycine performed at room temperature in the presence of (*1*) Na_2SO_3, (*2*) KCN (the reaction conditions see in Table 1), and (*3*) 2ME (the reaction conditions see in [5]).

At the same time, the initial concentration of the reagents should be sufficiently high to attain the required rate and extent of conversion of the amino acid to a fluorescent derivative. In our work, we optimized the following parameters: initial concentrations of OPA and nucleophilic agents and the reaction time from mixing the reagents to the end of the measurement of the fluorescence intensity or absorbance. The effect of pH on the analytical signal was also studied. The difference between the absorbance or fluorescence intensity of a glycine solution of a known concentration and those of a blank solution served as the analytical signal.

Optimum concentration of OPA. It is well known (see, for example [10]) that aromatic aldehydes readily form addition products with nucleophiles, including sulfite and cyanide ions. The absorbance spectra of reaction products overlap with those of substituted isoindols and, thus, interfere with the determination of amines and amino acids by their reaction with OPA. It was found that OPA taken in an equimolar ratio to the nucleophilic agent under consideration forms compounds absorbing in the region 300–340 nm. This effect was significant at OPA concentrations higher than 4×10^{-4} M, where the absorbance of the blank solution was too high. Taking this fact into account, we selected the OPA concentration of 2×10^{-4} M for the further experiments.

Optimum concentrations of nucleophilic agents and optimum reaction time. The absorbances and fluorescence intensities of the product of reaction of OPA with glycine as functions of nucleophilic agent concentrations with allowance made for blank experiments were obtained at the fixed OPA concentration (2×10^{-4} M) in the reaction mixture. The concentrations of KCN and Na_2SO_3 in solutions varied from 5×10^{-5} to 2×10^{-2} M. The data obtained are presented in Fig. 2 and Table 1. The similar kinetic curves built for the reaction of 2ME under the conditions reported in [5] showed a much higher stability of sulfo- and cyano-substituted isoindols (Fig. 3).

Table 2 presents analytical characteristics of the developed procedure with KCN and Na_2SO_3 as nucleophilic agents and those of the commonly used procedure with 2ME as a nucleophilic agent.

Thus, it was demonstrated that the analytical characteristics of the developed procedure were highly competitive with those of a widely used procedure based on 2-mercaptoethanol, whereas the stability of the analytical form (substituted isoindols) was much higher.

Table 2. Comparison of the analytical characteristics of procedures for the determination of glycine by its reaction with OPA in the presence of different nucleophilic agents

Nucleophilic agent	Wavelengths of absorption/fluorescence maxima (nm)	Parameters of the equation for the calibration graph, total analytical signal $y = a + bx$ in the fluorimetric determination ($P = 0.95$)	Detection limits (M)	
			fluorimetry	spectrophotometry*
2-Mercaptoetanol	340/450	$a = 0.10 \pm 0.03$ $b = (25.1 \pm 0.5) \times 10^4$	1.4×10^{-7}	–
KCN	333/385	$a = 0.24 \pm 0.07$ $b = (66.7 \pm 1.4) \times 10^4$	4.0×10^{-8}	1.0×10^{-7} (1.25×10^4)
Na_2SO_3	328/400	$a = 0.24 \pm 0.02$ $b = (30.7 \pm 1.4) \times 10^4$	4.8×10^{-8}	2.2×10^{-6} (3.6×10^3)

* In parentheses, extinction coefficients are given.

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