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A simple photokinetic method for the determination of nitroprusside in biological and pharmaceutical samples

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Abstract: A simple method has been developed for the determination of nitroprusside in human serum and pharmaceutical preparations. The new method is based on the strong inhibitory effect of nitroprusside on the photochemical reduction of phloxin by ethylenediaminetetra-acetic acid. The rate measurements are accomplished very simply by measuring the time needed for the absorbance to be reduced to 1/10th of its initial value. Optimal conditions for the determination of nitroprusside at concentrations of 15–200 ng ml $^{-1}$ are described.

Keywords: *Nitroprusside; inhibition; photokinetic method.*

Introduction

Pharmaceutical interest in nitroprusside (NP) was at first based on its application as an analytical reagent in pharmaceutical analysis (detection of sulphides, ketone bodies in urine, primary and secondary aliphatic amines, etc.), but recently interest has been focused on its strong hypotensive action [1]. NP is administered intravenously in hypertensive emergencies, to improve heart function after myocardial infarction and for controlled hypotension during surgery.

The toxicity of nitroprusside is due to its possible decomposition in cyanide [2]. In this process one electron is transferred from haemoglobin iron to NP resulting in the formation of methaemoglobin and cyanide [3]. However, it is generally accepted that the five cyanide ligands of NP are quickly released *in vivo* and partially converted into the relatively non-toxic thiocyanate. This transformation is mediated by hepatic and renal rhodanase, which is a sulphuryl transferase [4].

The polarographic activity of NP has been used as a basis for its quantification. Conventional and sine wave polarography, cyclic voltammetry and differential pulse polarographic methods have been described [5–11]. Flow-injection analysis with amperometric detection has also been used [12].

Spectrophotometric methods based on the reaction of NP with nucleophilic agents to form highly coloured compounds of satisfactory stability have been used [13–15], but most of these methods are associated with an unsatisfactory limit of detection and with an inability to determine NP selectively in serum, plasma or whole blood. In the reaction between NP and cysteine in the presence of methaemoglobin HCN is evolved and spectrophotometrically quantified [16]. NP has also been evaluated using the reaction with sulphide ion in an alkaline medium [17]. A method for the determination of NP in low concentration is based on the catalytic effect of nitroprusside on the Indophenol Blue reaction between ammonium, phenol and hypochlorite [18].

High-performance liquid chromatography (HPLC) and ^{13}C NMR methods have been used for the determination of NP in blood [19, 20].

The present paper reports a new method for the determination of nitroprusside at very low concentrations. It has been found that the photochemical reduction of phloxin by ethylenediaminetetra-acetic acid (EDTA) is strongly inhibited by even amounts of NP; this inhibitory effect can be used for the determination of traces of NP. The procedure has been successfully applied to the determination of NP in pharmaceutical preparations and blood serum.

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Experimental

Apparatus

The illumination device was very simple. An electronic voltage regulator was used to obtain a close voltage control for a stable radiation source. A Sylvania halogen lamp (250W, 24V) was used as the source of visible radiation. The light was passed through a small water-cooled chamber. A lens system was used to focus the light on the reaction cell, which was kept at 25 ± 0.5°C. A magnetic stirrer was used to stir the solution in the cell. The reduction of phloxin was followed with a photometric titration unit (EEL, Univalvo, 200). A Pye Unicam SP8-200 spectrophotometer was used for recording spectra.

Reagents

Analytical-reagent grade chemicals and double-distilled water were used. Aqueous 0.001 M phloxin (tetrachlorotetrabromofluorescein, C.I. 45410) was prepared from the commercial substance (Geigy). Aqueous 0.001 M sodium nitroprusside was prepared from the Merck substance and stored in a dark bottle. Aqueous 0.1 M EDTA was prepared.

General procedure

To 2 ml of 2 M acetate buffer (pH 5.7), 3 ml of 0.1 M EDTA and 2 ml of 1.5×10^{-4} M phloxin in a 20-ml volumetric flask was added an appropriate volume of nitroprusside solution (standard or sample) to give a final NP concentration of 15–200 ng ml^{-1}. The solution was diluted to 20 ml with water and transferred to the reaction cell, maintained at 25 ± 0.5°C. Oxygen was removed from the solution by bubbling pure (99.99%) nitrogen through the solution for 15 min. The halogen lamp was switched on and the time needed for the absorbance to be reduced to 1/10th of its initial value (from 0.920 to 0.092) was measured with a photometric titration unit.

A calibration graph was constructed by plotting nitroprusside concentration versus t_x/t_0, where t_x is the time required for photoreduction of the sample, and t_0 the time required for a nitroprusside-free sample. The illumination intensity was chosen to give a value of about 100 s for t_0.

Determination of nitroprusside in real samples

The sample of the pharmaceutical preparation was dissolved in double-distilled water; a suitable aliquot was analysed by the general procedure.

For blood serum, the sample was treated with perchloric acid to separate the proteins. After centrifugation, saturated potassium acetate solution was added. The precipitate was separated and NP was determined in the filtrate by the general procedure.

Results and Discussion

When a solution containing phloxin and EDTA in the absence of oxygen is illuminated at a suitable pH, photoreduction of the dye occurs and the pink colour disappears:

$$\text{Phloxin}_{ox} + \text{EDTA} \xrightarrow{h\nu} \text{Phloxin}_{red} + \text{EDTA oxidation products.}$$

The reaction proceeds at an adequate rate only if the light is sufficiently intense. If air is passed through the colourless solution, the dye is oxidized very quickly and the solution returns to its original pink colour. Figure 1 shows the absorption spectra of the dye before photoreduction and then after oxidation with oxygen or hydrogen peroxide of the leuco-phloxin formed by photoreduction. Since the three spectra coincide, it is concluded that phloxin does not undergo irreversible breakdown during the photochemical reaction.

The stoichiometry was determined by adding an excess of EDTA, at various pH values, photolysing until the dye was completely de-

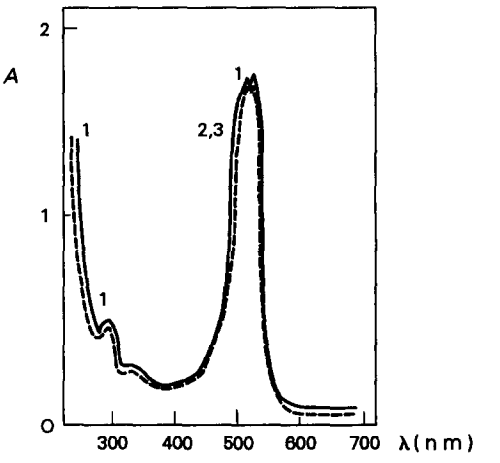

Figure 1
Absorption spectra for 2×10^{-5} M phloxin with 2×10^{-2} M EDTA in acetate buffer (pH 5.7). Curve 1: before the photochemical process. Curves 2 and 3: after the photochemical process and reoxidation with oxygen and hydrogen peroxide, respectively.

colorized, and titrating the remaining EDTA with zinc solution. The molar ratio of phloxin–EDTA was 1:1.

Effect of experimental variables

The rate of photoreduction of phloxin by EDTA is pH dependent, as shown in Fig. 2 (curve 1).

The initial rate and integration techniques were used for determination of the order of reaction with respect to phloxin. For both methods the absorbance of the solution was monitored with photolysis time. The results (Fig. 3 and Table 1) of both methods show a first-order dependence on phloxin concentration.

The overall reaction order was also determined by the integration method. The results in Table 2 show an overall reaction order of 2; thus the reaction is also first-order with respect to EDTA.

Variations in temperature between 20–60°C were shown to have very little influence on the rate of the photochemical process.

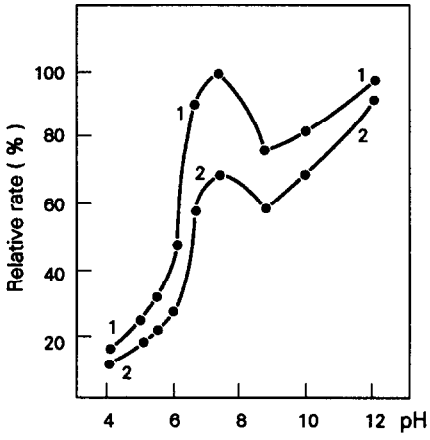

Figure 2
Rate of photoreduction as a function of pH. Curve 1: without NP. Curve 2: [NP] = 3 × 10^{-6} M.

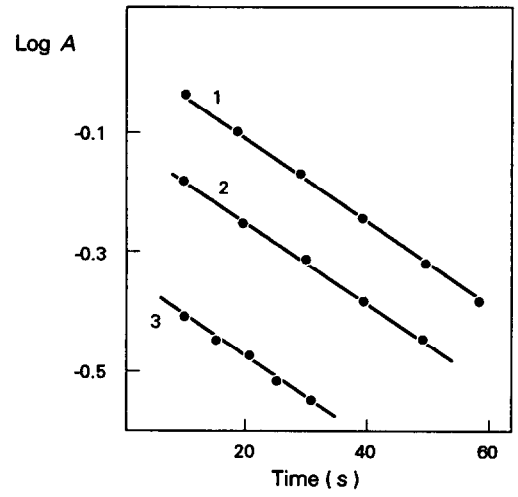

Figure 3
Plots of the logarithm of absorbance against time. EDTA 3.3 × 10^{-2} M; acetate buffer (pH 5.7); T = 25°C. Curve 1: phloxin = 1.25 × 10^{-5} M. Curve 2: phloxin = 9.0 × 10^{-6} M. Curve 3: phloxin = 5.0 × 10^{-6} M.

Table 1
Values of the experimental pseudo-first-order constant, k, for different initial concentrations of phloxin*

[Phloxin] (μM)	k (min^{-1})
10.0	0.71
9.0	0.85
8.0	0.68
7.0	0.82
6.0	0.70
5.0	0.85

*[EDTA] = 3.3 10^{-2} M; pH = 5.7; T = 25°C.

Study of the photochemical reaction in the presence of nitroprusside

It was found that the photochemical reduction of phloxin is strongly inhibited by the presence of NP, even in small amounts. However, there are a number of published reports

Table 2
Values of the experimental overall second-order constant, k*

Photolysis time (s)	Absorbance	[$Phloxin_{red}$] (μM)	k l mol^{-1} in^{-1}
180	0.973	0.56	22.8
360	0.927	1.11	24.9
540	0.860	1.94	28.2
720	0.814	2.50	27.9
900	0.769	3.06	28.1

*a = [$Phloxin_{ox}$] = 1.25 10^{-5} M; b = [EDTA] = 6.67 10^{-4} M; pH = 5.7; x = [$Phloxin_{red}$];

$$k = \frac{1}{t(a-b)} \ln \frac{b\,(a-x)}{a\,(b-x)}.$$

about the photodecomposition of NP. In solution NP is extremely photosensitive and degrades rapidly by numerous reactions. The literature descriptions of the photodecomposition pathways are contradictory. The two most cited pathways of photochemical degradation have been formulated by Mitra and others [21, 22], on the one hand, and by Buxton [23], and Wolfe and Swinehard [24] on the other.

In the most recent literature the studies of Jarzynowski [25, 26] are important. Their conclusions were that: irradiation in the band at 498 nm does not result in photodegradation; irradiation in the region of 396 nm produces hydration; and irradiation in the region of bands at 265 nm and shorter wavelengths produces photo-oxidation and replacement of NO by H_2O.

The photochemistry of NP is interesting not only from a theoretical point of view but also in connection with its use as a potent hypotensive agent. When protected from light, the concentrated solution for injection is stable for more than 2 years. The stability of the NP solution is enhanced by the following conditions: pH 3–6; exclusion of oxygen; avoidance of temperature increase; and addition of iron complexing agents [7]. The experimental conditions in the present work are: pH 5.7; nitrogen atmosphere; controlled temperature; and an excess of EDTA. Under these conditions NP, although exposed to radiation from the halogen lamp, does not undergo photochemical reaction.

On the other hand, the photoreactive state of phloxin is probably the triplet since EDTA does not quench the fluorescence of phloxin although it is oxidized by the activated dye. Furthermore, small amounts of NP do not quench the fluorescence of the dye but dramatically retard the photochemical reaction. Retardation is due to an energy-transfer process between the inhibitor and phloxin in the triplet state, which has a relatively long life.

In accordance with these results it can be assumed that inhibition is the only effect caused by NP on the photochemical process of the phloxin–EDTA system. Inhibition is due to the interaction of NP with the triplet state of phloxin; thus reduction of the dye by EDTA is hindered.

There are a number of processes such as inter-system crossing, energy transfer or chemical reaction, whereby absorbed radiant energy can be dissipated by an excited-state molecule in its return to the initial ground state. Each process has an associated rate constant. When the steady-state hypothesis is applied in kinetic schemes with and without inhibitor, an expression similar to that of Stern-Volmer [27, 28] is obtained:

$$\frac{\Phi^0}{\Phi} = 1 + \frac{k_e[\mathrm{NP}]}{k_d + k_r\,[\mathrm{EDTA}]}, \qquad (1)$$

where Φ^0 and Φ are the quantum yields in the absence and presence of NP, respectively; k_e is the rate constant of the energy transfer (quenching) of the triplet state of the phloxin to the NP; k_d is the overall rate constant of intramolecular deactivation of the triplet state of phloxin (phosphorescence and inter-system crossing); and k_r is the rate constant of the photochemical reaction.

For measurements made with a fixed light intensity:

$$\frac{\Phi^0}{\Phi} = \frac{V_0}{V_x} = 1 + \frac{k_e[\mathrm{NP}]}{k_d + k_r[\mathrm{EDTA}]}, \qquad (2)$$

where V_x and V_0 are the reaction rates with and without NP, respectively.

With a large excess of EDTA so that its concentration can be deemed constant:

$$\frac{V_0}{V_x} = 1 + k\,[\mathrm{NP}]. \qquad (3)$$

This equation enables NP to be determined by measurement of the rate of the photochemical process in its presence and absence.

The reaction conditions need to be optimized. Curve 2 of Fig. 2 shows the variation in the rate of the photochemical process in the presence of NP at several pH values. In Fig. 4 the inhibitory effect of NP is plotted as a function of pH.

Acetate buffer (pH 5.7) gives a high degree of inhibition coupled with a moderate rate of photoreduction.

Figure 5 shows that the rate of the photochemical reaction is enhanced as the EDTA concentration is increased up to 1.2×10^{-2} M; above this concentration of EDTA there is no further increase in the rate of reaction.

The rate of photoreduction of phloxin by EDTA in the presence of NP is affected by temperature. Table 3 lists the photoreduction

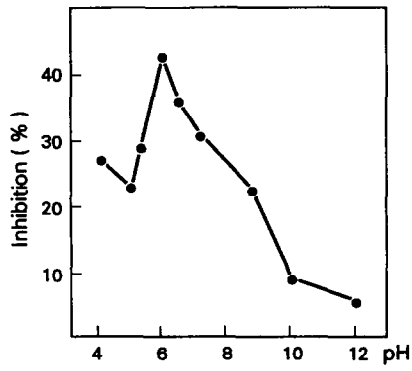

Figure 4
Percentage inhibition as a function of pH.

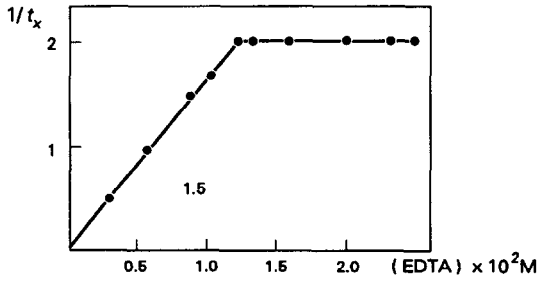

Figure 5
Influence of EDTA concentration on the rate of the photochemical process. [Phloxin] = 1.5×10^{-5} M; [NP] = 3×10^{-6} M; and pH = 5.7.

times for identical samples at different temperatures.

To summarize, the best experimental conditions for the determination of NP are: EDTA concentration = 1.5×10^{-2} M; phloxin concentration = 1.5×10^{-5} M; pH = 5.7; and temperature = 25 ± 0.5°C.

Table 3
Dependence on temperature of the photoreduction of phloxin by EDTA

Temperature (°C)	Photolysis time (s)
25	287
30	280
35	273
40	270
50	240
60	165

Nitroprusside determination

Equation (3) shows that NP can be determined by means of measurement of the rate of photoreduction of phloxin if the variables of the photochemical process are controlled. It was decided to use the variable time method, in which the time required for the sample to reach a preselected absorbance was measured.

The concentration range for the determination of NP is 7.0×10^{-8}–9.2×10^{-7} M. For higher levels of NP the photolysis times are too long and it is advisable to dilute the sample. A study of the precision was performed by carrying out 10 independent measurements on solutions of various concentrations of NP and fixed concentrations of EDTA, phloxin and acetate buffer. The relative standard deviation was 1.64–2.02% for NP at concentrations of 6.9×10^{-7}–2.3×10^{-7} M. The lower limits of detection and quantification are 10^{-8} M and 5.7×10^{-7} M, respectively.

Interference

Table 4 shows the interference of diverse ions or substances on the determination of NP. The limiting value of the concentration of a foreign ion or substance was taken as that

Table 4
Interference of diverse ions and substances with the determination of nitroprusside

Ion or substance	Limiting ratio of added ion or substance to NP
Glucose, citrate, NO_3^-, SO_4^{2-},	2000†
Br^-, Ba(II), Cl^-	750
ClO_4^-	500
PO_4^{3-}, Zn(II), Cd(II), Pb(II), Ca(II)	100
Mg(II)	50
I^-, Cr(III), Co(II)	10
Fe(III), Ni(II)	5
Cu(II)	1
Ag(I), Hg(II)	0.2

*Concentration of $[Fe(CN)_5NO]^{2-}$ = 99.3 ng ml^{-1}.
†Maximum molar ratio tested.

Table 5
Determination of nitroprusside in pharmaceutical preparations*

Sample	Source	Amount declared (mg)	Amount found (mg)†
Sodium nitroprusside solution	Fides	50	50.12

*Composition of sample solution; sodium nitroprusside, 50 mg; sodium citrate, 45 mg; glucose, 250 mg; double-distilled water to 5 ml.
†Mean of four determinations.

Table 6
Determination of nitroprusside in blood serum

	NP added (ng ml^{-1})	NP found* (ng ml^{-1})
Sample 1	49.64	48.29
Sample 2	99.31	99.07
Sample 3	148.98	146.77

*Mean of four determinations.

value which caused an error of not more than 2% in the assay. If metal ions that form stable EDTA complexes are present, preliminary addition of EDTA is required; sufficient EDTA must be added to the test sample to fix the metal ions as chelates and to leave a sufficient amount of free EDTA.

Application

The method has been applied to the determination of NP in pharmaceutical preparations. Table 5 shows the results obtained; these are in excellent agreement with those found by a polarographic method [8].

The method has also been applied to the determination of NP in blood serum. The sample of blood serum was collected from a healthy volunteer and NP was added at a similar concentration to those used in clinical applications. The results (Table 6) show excellent recoveries of NP.

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