

A SERIES OF NEW DYES FOR CENTRAL NERVOUS SYSTEM SECTIONS STAINING. NOTE 2

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Abstract

The new diazo dye 5-[3-[(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)carbamoyl]-4-hydroxy-phenyl]azo-2-[2-[4-[3-[(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)carbamoyl]-4-hydroxy-phenyl]azo-2-sulfo-phenyl]vinyl] benzenesulfonic acid (called SINT 8) was obtained by synthesis using 4,4'-diaminostilbene-2,2'-disulfonic acid (called AASS) and N-(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)-2-hydroxy-benzamide. The Diazo component (AASS) and the coupling component were likewise obtained by synthesis. [1]. A similar diazo dye has not been prepared before, probably due to the difficulty of its synthesis.

The new component was purified and analysed by IR and NMR spectrometry and elemental analysis, in order to confirm the proposed structure [1]. The tests proved that the new diazo dye had antimicrobial effects [2] (similar to other bibenzoderivatives [3]) and remarkable staining properties [4], [5], [6].

This study reports the results of HPLC and spectrometric UV-VIS analysis, correlates the structure and analytical properties with the affinity for different areas of the central nervous system.

HPLC analysis with diode array detector revealed the presence of two components. Overlaid spectra of each peak demonstrated the absence of synthesis precursors and the purity of the diazodye. Solvatochromic and photoisomerization studies correlated with other determinations lead to the conclusion that the two separated components are Z and E isomers of the diazo dye [4].

UV-VIS spectrophotometric studies were used to determine ionization constant values and spectral properties.

The tests demonstrated the special staining properties of the new diazodye. Human central nervous system sections (cerebellum cortex, cerebral cortex and spinal cord) stained with this new diazo dye clearly reveal each structure.

Rezumat

A fost sintetizat un nou colorant diazoic, acidul 5-[3-[(1,5-dimetil-3-oxo-2-fenil-pirazol-4-il)carbamoil]-4-hidroxi-fenil]azo-2-[2-[4-[3-[(1,5-dimetil-3-oxo-2-fenil-pirazol-4-il)carbamoil]-4-hidroxi-fenil]azo-2-sulfo-fenil]vinil] benzensulfonic (denumit SINT 8) folosind acidul 4,4'-diaminostilben-2,2'-disulfonic (denumit AASS) și N-(1,5-dimetil-3-oxo-2-fenil-pirazol-4-il)-2-hidroxi-benzamida, drept componentă de cuplare. Componenta de diazotare (AASS) și componenta de cuplare au fost obținute tot prin sinteză [1]. Acest compus nu s-a preparat până acum, posibil datorită dificultăților de sinteză ale unui colorant diazoic.

Noul component a fost purificat și supus analizei IR, RMN și analizei elementale pentru confirmarea structurii propuse [1]. Testele au dovedit că noul colorant diazoic are efecte antimicrobiene [2] (similar altor dibenzoderivați [3]) și remarcabile proprietăți de marcă histologică [4], [5], [6].

Studiul prezintă rezultatele analizei cromatografice HPLC și spectrofotometrice UV-VIS cu scopul de a corela proprietățile analitice cu afinitatea evaluată pentru secțiunile sistemului nervos central.

Analiza cromatografică HPLC cu detector cu șir de diode a relevat prezența a doi componenți. Spectrele fiecărui *peak* separat suprapuse cu spectrele componentei de diazotare și componentei de cuplare au dovedit absența precursorilor de sinteză și puritatea colorantului diazoic. Studiile de solvatocromie și fotoizomerizare corelate cu celelalte determinări conduc la concluzia că cei doi componenți separați sunt izomerii Z și E ai noului compus de sinteză [4].

Studiile spectrofotometrice UV-VIS s-au aplicat pentru determinarea constantelor de ionizare și a proprietăților spectrale.

Testele au demonstrat proprietățile deosebite de marcă a secțiunilor histologice. Secțiunile sistemului nervos central uman (cortex cerebelos și cerebral și măduva spinării) marcate cu acest colorant au pus în evidență toate elementele sale structurale.

Keywords: azodye; histological dye; staining; biological marker

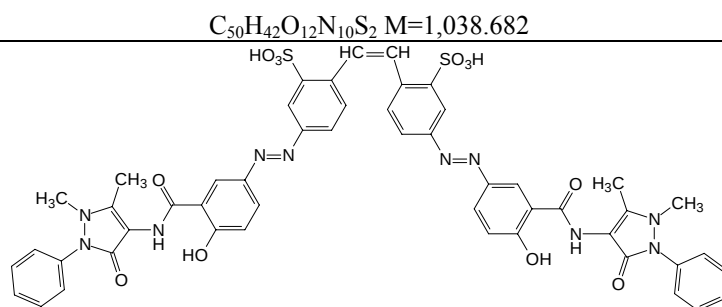
Introduction

The purpose of this project is the study of the new azo dye 5-[3-[(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)carbamoyl]-4-hydroxy-phenyl]azo-2-[2-[4-[3-[(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)carbamoyl]-4-hydroxy-phenyl]azo-2-sulfo-phenyl]vinyl]benzenesulfonic acid (called SINT 8) derived from 4,4'-diaminostilbene-2,2'-disulfonic acid and the evaluation of its staining properties.

The synthesis was conducted using the 4,4'-diaminostilbene-2,2'-disulfonic acid as azo-component and N-(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)-2-hydroxy-benzamide, as coupling component. The structure of the 4,4'-diaminostilbene-2,2'-disulfonic acid allows the presence of two isomers: Z- and E-isomers. The derived azo dye also has two isomers (Figure 1).

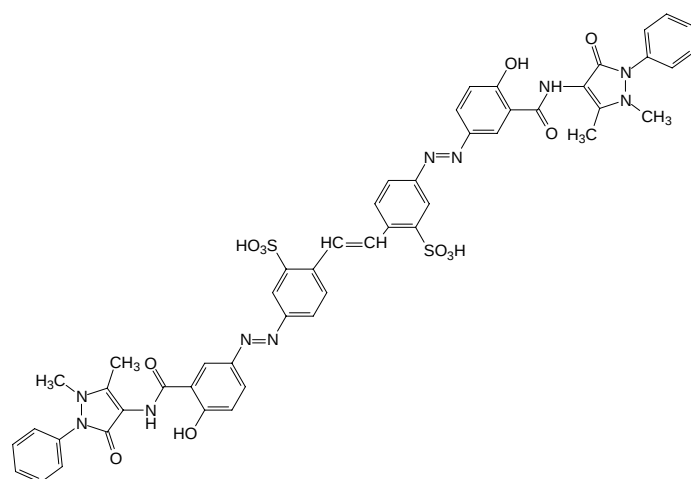
Materials and methods

The fundamental analytical study of diazo dye 5-[3-[(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)carbamoyl]-4-hydroxy-phenyl]azo-2-[2-[4-[3-[(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)carbamoyl]-4-hydroxy-phenyl]azo-2-sulfo-phenyl]vinyl]benzenesulfonic acid (SINT8) followed two directions: HPLC and spectrophotometric UV/VIS analysis.



Z isomer

5-[3-[(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)carbamoyl]-4-hydroxy-phenyl]azo-2-[(Z)-2-[4-[3-[(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)carbamoyl]-4-hydroxy-phenyl]azo-2-sulfo-phenyl]vinyl]benzenesulfonic acid



E isomer

5-[3-[(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)carbamoyl]-4-hydroxy-phenyl]azo-2-[(E)-2-[4-[3-[(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)carbamoyl]-4-hydroxy-phenyl]azo-2-sulfo-phenyl]vinyl]benzenesulfonic acid

Figure 1

Z and E isomers of

5-[3-[(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)carbamoyl]-4-hydroxy-phenyl]azo-2-[2-[4-[3-[(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)carbamoyl]-4-hydroxy-phenyl]azo-2-sulfo-phenyl]vinyl]benzenesulfonic acid (called SINT 8)

The HPLC method was carried out using the GBC-HPLC instrumentation with the following configuration: LC 1150 Quaternary Solvent Delivery System, LC 1460 Vacuum On-Line Degasser, LC 1445 System Organiser, LC 1150 Column Oven, LC 5000 Photo Diode Array Detector (DAD with the range 190-800 nm), WinChrom Chromatography

Data Management System, Column RP C₁₈, 150x4.6mm, (Reverse Phase Octadecyl Sylane), 5 µm, guard column 30x4.6 mm, RP C₁₈, 5 µm.

The aim of the HPLC analysis was the separation, identification and determination of each chemical species present in the sample solution. Therefore, a stock solution of diazo dye in methanol 50% and a series of working solutions by dilution of the stock solution to 10-70mg/L (ppm) were prepared.

For optimum separation, 12 chromatographic methods were tested, called Sint1.met.-Sint12.met. The method with the best result was applied in the diazodye and precursors of the synthesis analysis. HPLC results are illustrated by Spectrum Max Plot, Spectrum Plot and Overlaid Spectra.

The Spectrum Max Plot generates a chromatogram, whereby each point is plotted at its maximum absorbance, indicating how the chromatogram will appear when the wavelength for each one is optimized.

Diode Array Detector allows spectra acquisition in real time for each peak detected. Overlaid spectra of the sample and standard peaks expressed by Similarity Index (SI) are used for identification of each separated component. The Similarity Index (SI) compares two normalized spectra across the wavelength range defined in the Library Search in Photo Diode Array Setup. The Similarity Index is a number between 0 and 1.

Spectrometric analysis is performed using CINTRA 10e GBC Spectrometer: Wavelength Accuracy ±0.2nm; Wavelength Reproducibility ±0.04nm; Photometric Accuracy ±0.002A; Photometric Repeatability ±0.0001A; Photometric Linearity Bbetter than 1% at 3 Abs; Wavelength Range 190-1,200nm; Spectral Bandpass 1.5nm; Scanning Speed 15,000nm/minute; module with external IBM Compatible computer; Photometric System double-beam; Light Source Tungsten-halogen lamp and deuterium lamp; Monochromator Czerny-Turner mounting; Detector Silicon Photodiode.

Spectrophotometric analysis was applied for absorption spectra of the solutions with the same pH and different concentrations, for calibration curve and for absorption spectra of the solutions with the same concentration and different pH determinations.

Absorption spectra of the solutions with the same pH and different concentrations in the range 190-1200nm, for 10 to 100 mg/L (ppm), reveal the wavelength of maximum absorbance and were correlated with the result of the chromatographic analysis.

The equation of the calibration curve was used for spectral properties computation: absorbtivity, specific absorbance $A_{1cm}^{1\%}$ and molar absorbtivity- ϵ . Absorption spectra of the solutions of the same concentration and different pH, were applied for determining ionization constants values.

Staining method

In order to visualize the nervous tissues, representative samples are specially prepared, which typically involves preservation and subsequent coloration (staining), prior to the microscopic examination.

Fixation is the rapid preservation of tissue components in order to arrest cellular processes and to maintain, as close as possible, a resemblance to the living condition (10% formalin: formaldehyde dissolved in an aqueous or buffered medium). Equally important, fixation also resists tissue degradation by endogenous (enzymatic autolysis) or exogenous (bacterial action) mechanisms.

Then a dehydration sequence is employed to gradually introduce an organic phase that will be compatible with the embedding medium. This sequence is a graded series of ethanol solutions (e.g., 50, 70, 95, 100%). Once absolute ethanol is achieved, the tissue is placed in an intermediate organic solvent that is miscible with both the alcohol and the wax (e.g., xylene, toluene, benzene, or other clearing agents). The opaque tissue specimens become clear when placed in some of these agents -“clearing”.

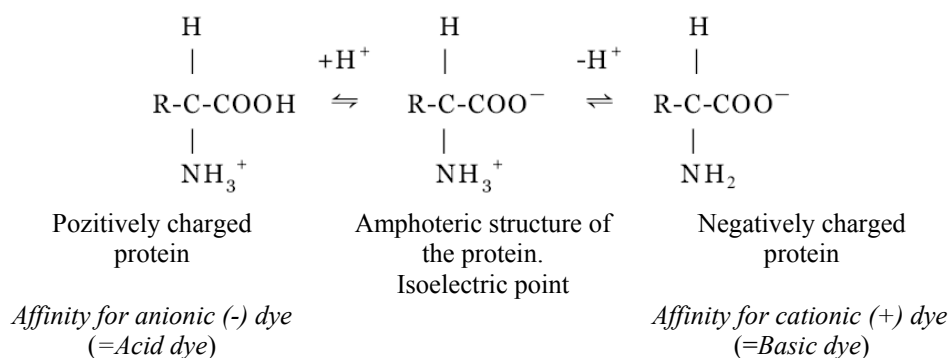
The specimen is then placed in several changes of molten wax, in order to replace the clearing agent and completely infiltrate the tissue with the wax embedding medium.

Embedded tissue is removed from its mold, trimmed, mounted and secured on a cutting instrument – the microtome. Thin slices of tissue (typically 2-10 μm in thickness) are obtained. These tissue sections are then mounted on glass slides for coloration.

In this study it was applied the hematoxylin-eosin technique, replacing eosin with the new azo dye.

Following staining procedures, tissue sections affixed to slides are covered by a thin glass disk or plate coverslip or cover glass. These coverings not only protect tissue specimens from the environment, but they also facilitate microscopic examination, micrography, and other manipulations.

Proteins are the substances that contribute in large measure to the stainability of tissue sections. Since proteins are amphoteric, they possess both acidic and basic groups. At the isoelectric point (pI ; see Fig. 2), the net charge is zero and minimal dye binding occurs. If staining takes place above the isoelectric point of a given protein, its acidic groups (phosphate, carboxyl, or sulfate) will be negatively charged and can combine with a positively charged cationic dye (Fig. 2 right).

**Figure 2**

Schematic representation of dye-protein interactions

At *pI* of protein (Fig.2 center), negligible binding of charged dyes occurs. Below *pI*, protein binds anionic dyes (=acid dye) (Fig.2 left).

At physiologic pH, specific proteins may exhibit net (+) or net (-) charges and are therefore characterized as acidophilic (=affinity for acid dyes) or basophilic (=affinity for basic dyes), respectively.

Dye solution used to stain biological sections is 0.25% in methanol and has a pH=4. At this pH, the dye studied is between the second and the third dissociation step. Anionic structure of the dye with one or two negative charges allows the binding to proteins with positive charges-the most probable ionization structure of the protein at this pH. This may be the explanation of different colours given to each component of the biological sections.

Results and discussion

Chromatographic analysis

Standard solution SINT8 (C=81 mg/L) in methanol 50% was analysed chromatographically (Sint8.met): Column RP C₁₈ 5μ 150x4.6 mm, Guard column RP C₁₈ 5μ, 50x4.6 mm; Mobile phase Methanol/water/acetonitrile/sodium pentanesulfonate=25/25/25/25 (v/v/v/v); Flow rate 1 mL/min; Temperature 30°C; Detector PDA (200-700 nm); units: mAU; Sample size 100 μL; Sample conc. 81 mg/L (methanol 50%)

This method yielded the best results. The chromatogram Spectrum Max Plot (Absorbance in mAU versus time in min.) reveals the presence of two components in almost equal parts: first 49.95% and the second 48.67%, very well separated (resolution 1.626) (Figure 3).

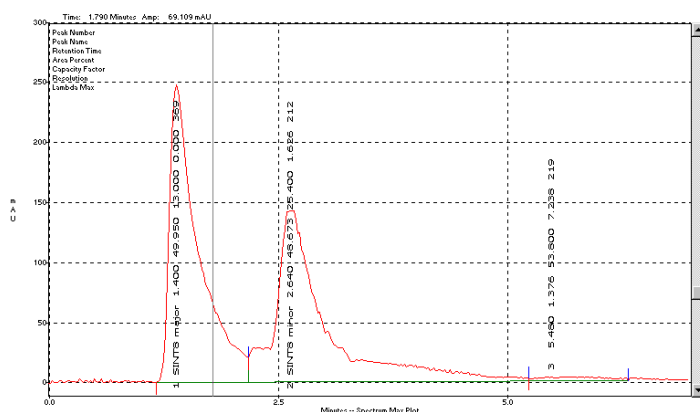
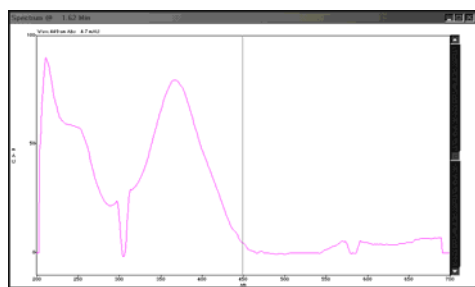
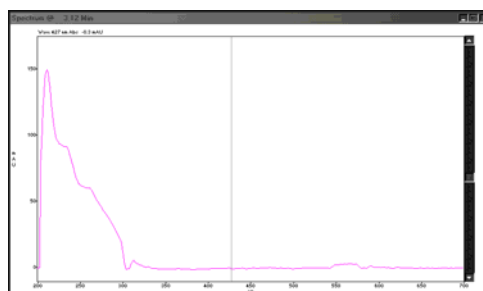


Figure 3
Spectrum Max Plot standard solution SINT8

The spectral profiles of the two components are quite different: the first component evidences a wide absorption band in the near VIS region (350-450 nm) with the maximum of absorbance at 369nm, whereas the second component has a maximum level of absorbance at 212nm. This difference allows the first component analysis in the range of 300-400nm, the second component manifesting unelectable or no absorbance. (Figure 4).



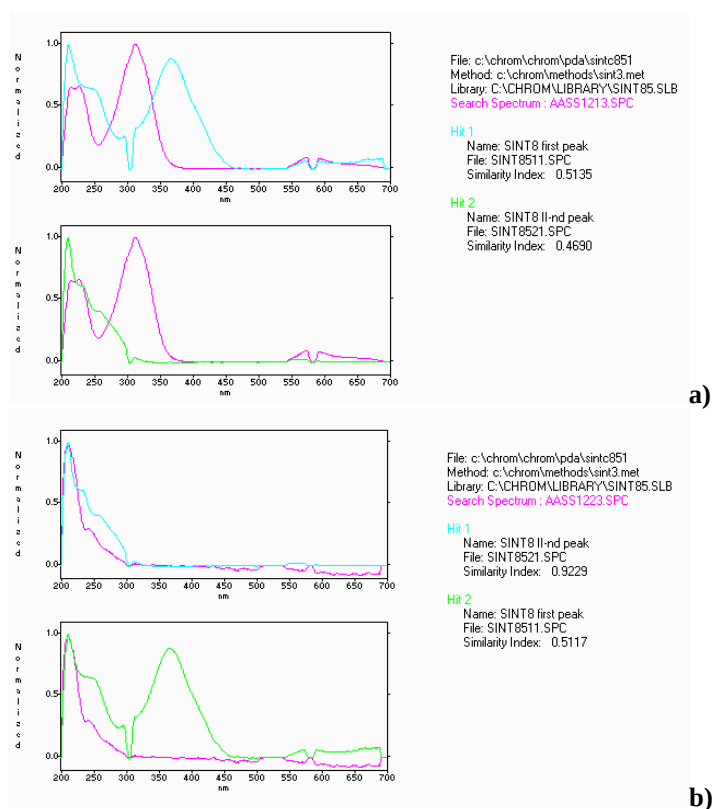
SINT 8 Spectral profiles of the I-st peak



SINT 8 Spectral profiles of the II-nd peak

Figure 4
Spectral profiles of the two SINT 8 components

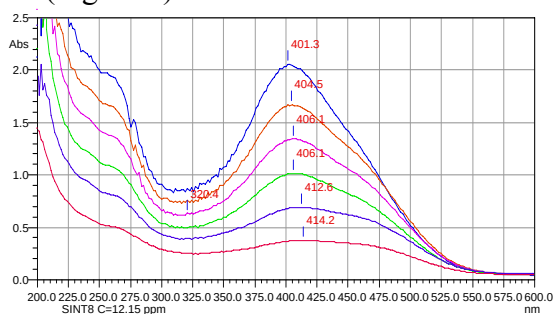
Overlaid normalized spectra of the two peaks of SINT 8 and spectral profiles of the p,p'-diaminostilbene-o,o'-disulfonic acid (AASS) and, on the other hand, spectral profiles of the N-(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)-2-hydroxy-benzamide turn out the absence of the two precursors in the investigated diazo dye. (Figure 5a and 5b).

**Figure 5**

Overlaid normalized spectra of the two peaks of SINT 8 and spectral profiles of the 4,4'-diaminostilbene-2,2'-disulfonic acid (AASS); a) first peak; b) second peak

Spectrophotometric analysis

Absorption spectra of standard SINT 8 solutions, in methanol 50%, with the same pH and concentration 10-73mg/L (ppm), exhibit maximum absorbances in near UV region and an absorption band in VIS with the maximum at 400nm (Figure 6).

**Figure 6**

Absorption spectra of standard SINT 8 solutions in the range of 200 -600nm (C=12-73 mg/L)

Spectrophotometric quantitative analysis

Calibration curves of standard SINT 8 solutions ($C_{\text{SINT 8}}=12-73\text{mg/L}$) demonstrate a very good correlation coefficient at the wavelength 400nm, according to Lambert-Beer law. Quantitative analysis at 400nm allows spectral properties determination for the first component separated, chromatographic analysis showing that the second one has no absorbance over 350nm.

Spectral parameter computation at 400nm:

Calibration curve equation: $A_{400\text{nm}} = 0.0277 \times C_{\text{SINT 8}}$
 $R^2=0.9977$

Absorbivity: $a = 0.027 \frac{L}{\text{mg} \times \text{cm}} = 27.7 \frac{L}{\text{g} \times \text{cm}}$

Specific absorbance: $A_{1\text{cm}}^{1\%} = 10 \times a = 277$

Molar absorbivity:

$\epsilon = a \times M = 27.7 \frac{L}{\text{g} \times \text{cm}} \times 1,038.682 \frac{\text{g}}{\text{mol}} = 28,044.4 \frac{L}{\text{mol} \times \text{cm}}$

Spectrophotometric determination of ionization constants

In order to determine ionization constants, 32 standard solutions SINT 8 of the same analytical concentration ($C=32.4\text{ mg/L}$) and different pH in the range 0-14 were examined spectrophotometrically.

The plot of absorbance of the certain wavelength versus pH in a narrow pH interval (a few units) or the plot of $\lg \frac{A_{\text{pH}}}{A_{0,25} - A_{\text{pH}}}$ versus pH in

a narrow pH interval, are the methods for spectrophotometric determination of ionization constants. Thus, three dissociation steps were identified [3]:

- In strong acidic medium, $\text{pH}=0.7-1.3$, isosbestic point at $\lambda=350\text{ nm}$, $\text{pK}_{\text{a},1}=0.8$.
- In acidic medium $\text{pH}=1.3-3.5$, isosbestic point at $\lambda=335$ and 456nm (Fig.7.a), $\text{pK}_{\text{a},2}=1.92$.
- In cvasineutral medium, $\text{pH}=4-5.5$, isosbestic point at $\lambda=425\text{nm}$ (Fig.7.b), $\text{pK}_{\text{a},3}=6.3$.
- In alkaline solution, $\text{pH}=10-12$, isosbestic point at $\lambda=525\text{nm}$, but experimental procedure for the ionization constant determination has no clear result.

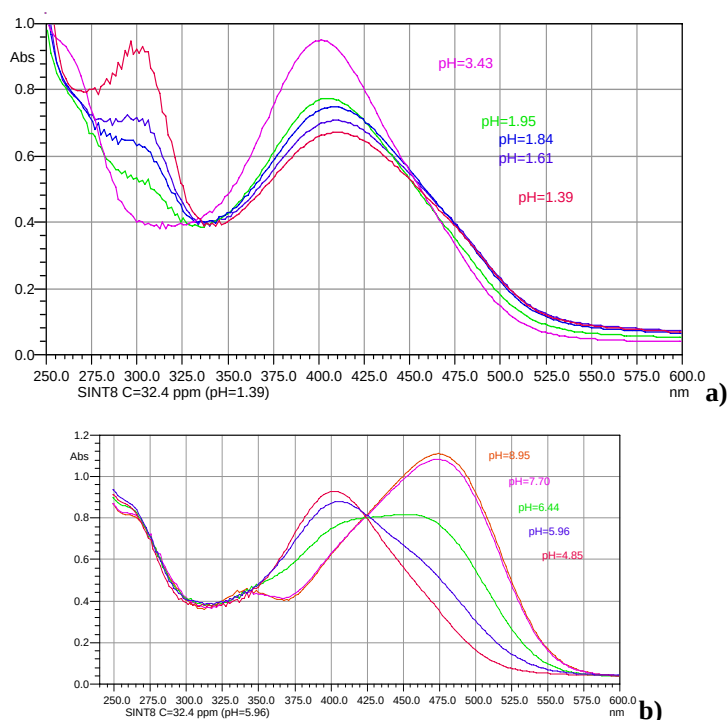


Figure 7

Overlaid absorption spectra of standard SINT8 solutions with the same analytical concentration ($C=32.4$ mg/L) and different pH, in the range 250-600nm, a). pH=1.3-3.5; b). pH=4.9-9

Central nervous system staining

Figure 8 presents a picture of Human cerebellum cortex section stained with SINT8 (Eyepiece Lens 10x, Objective Lens 10x). We noted the following:

- **Medullary core:** The white matter of the cerebellum contains nerve fibers that carry the afferent input and efferent output of the cerebellum
- **Granular layer:** Closely packed with the nuclei of small neurons (granule cells) colored in blue. Receives the major input to the cerebellum (the mossy fiber input). **Purkinje cell:** Single row of large flask-shaped neurons, red-brown. Dendrites arborize richly in the molecular layer. Axons exit in the medullary core.
- **Basket cell processes:** Axons of basket cells run Eversely in the folium giving off collaterals that form a basket-like arborization around Purkinje cell bodies. They also synapse with Purkinje cell dendrites and proximal parts of the axon.
- **Molecular layer:** This is the most superficial layer. It is sparsely cellular and is largely a synaptic layer. It is primarily composed of

the dendrites of Purkinje, stellate, and basket cells and axons of granule cells.

Figure 9 presents a Human cerebral cortex section stained with SINT8 (Eyepiece Lens 10x, Objective Lens 10x) reveals closely packed small neurons, axons and dendrites of cells.

This is a section from the cerebral motor cortex, where the pyramidal cells are found. Note the large multipolar cell body (perikaryon) and the apical dendrite directed toward the surface of the cortex. Surrounding these nerve cell bodies are processes of neural and glial origin (neuropil). In the neuropil, innumerable synaptic contacts occur between nerve cells and their processes.

Figure 10 is a section of Human spinal cord stained with SINT8 (Eyepiece Lens 10x, Objective Lens 10x).

Note the variation in axon diameter. The diameter of a nerve fiber is directly related to the speed of nerve impulse Emission; larger fibers carry electrical impulses faster than smaller fibers.

The staining of human central nervous system sections

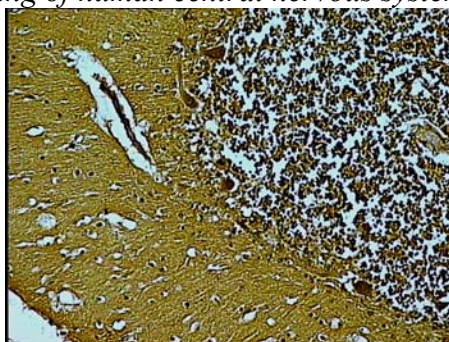


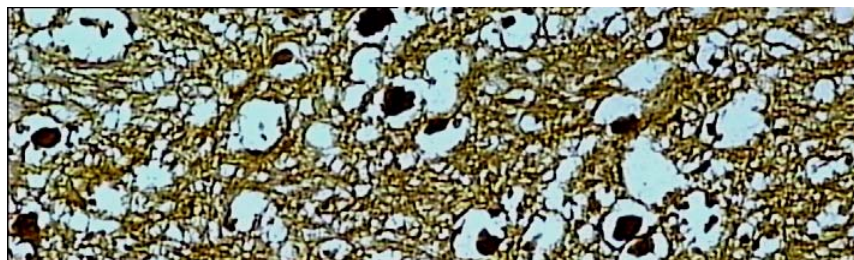
Figure 8

Human cerebellum cortex section stained with SINT8 (Eyepiece Lens 10x, Objective Lens 10x)



Figure 9

Human cerebral cortex section stained with SINT8 (Eyepiece Lens 10x, Objective Lens 10x)

**Figure 10**

Human spinal cord stained with SINT8 (Eyepiece Lens 10x, Objective Lens 10x)

Conclusions

The new diazoic dye obtained by synthesis, 5-[3-[(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)carbamoyl]-4-hydroxy-phenyl]azo-2-[2-[4-[3-[(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)carbamoyl]-4-hydroxy-phenyl]azo-2-sulfo-phenyl]vinyl]benzenesulfonic acid (called SINT 8) was analyzed by HPLC and spectrophotometric methods and tested for histological applications.

The HPLC methods revealed the presence of two components and the absence of the synthesis precursors. The first component displays a large absorption band in the VIS region and the second, a narrow band in UV. Since conjugation of chromophores and auxochromes is better in the E isomer, absorbance shifts to a greater wavelength. Thus, the bathochromic effect, solvatochromic studies and photoisomerization tests [4] admit identifying the component with absorption band in VIS region-E isomer.

The spectrophotometric method focused on two directions: spectral parameters computation (absorbivity, specific absorbance $A_{1cm}^{1\%}$ and molar absorbivity ϵ) in near VIS region, meant for E isomer and ionization constants determination for the first three dissociation steps.

Central nervous system staining

The study of the neural tissue is facilitated by several stains, none of which is exclusively capable of revealing all the desired details of structure. Due to the affinity of nerve cells for silver solutions (argyrophilia), silver impregnation methods are frequently used to demonstrate them [7].

The new azo dye was studied as a histologic dye for central nervous system providing good staining properties for cerebellum cortex, cerebral cortex and spinal cord. These tissue sections are usually stained using expensive reagents (silver and gold salts) and time consuming technique. The methods are coming from the beginning of XXth century. The new method is simpler, the reagents are accessible and the results are very good.

Acknowledgments

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- The staining procedure was carried through in the Cytodiagnostic Laboratory of the “Ovidius” University of Constanta Faculty of General Medicine, with the professional support of prof. Teofil Mehedinti, MD, PhD.