

A SERIES OF NEW DYES FOR CENTRAL NERVOUS SYSTEM SECTIONS STAINING. NOTE 1

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Abstract

The new diazo dye 5-[4-hydroxy-3-[[4-(thiazol-2-yl sulfamoyl)phenyl] carbamoyl] phenyl] azo-2-[2-[4-[4-hydroxy-3-[[4-(thiazol-2-ylsulfamoyl) phenyl] carbamoyl] phenyl] azo-2-sulfo-phenyl]vinyl]benzenesulfonic acid (called SINT 7) were obtained by synthesis using 4, 4'-diaminostilbene 2,2'-disulfonic acid (called AASS) and 2-hydroxy-N-[4-(thiazol-2-yl sulfamoyl) phenyl] 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 prove that the new diazo dye shows microbiologic effects [2] (similar to other bibenzoderivatives [3]) and remarkable staining properties [4], [5].

This study reports the results of HPLC and spectrometric UV-VIS analysis trying to correlate 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 separated with those of the diazo and coupling components demonstrate the absence of synthesis precursors and the purity of the diazo dye. 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 diazo dye. Human central nervous system sections stained with this new diazo dye clearly reveal each structure.

Rezumat

Noul colorant diazoic acidul 5-[4-hidroxi-3-[[4-(tiazol-2-il sulfamoil)fenil] carbamoil] fenil] azo-2-[2-[4-[4-hidroxi-3-[[4-(tiazol-2-ilsulfamoil) fenil] carbamoil] fenil] azo-2-sulfo-fenil]vinil]benzensulfonic (notat SINT 7) a fost sintetizat folosind acidul 4, 4'-diaminostilben 2,2'-disulfonic (notat AASS) și 2-hidroxi-N-[4-(tiazol-2-il sulfamoil) fenil] 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.

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 unor dibenzoderivați [3]) și remarcabile proprietăți histologice [4], [5].

Studiul prezintă rezultatele analizei cromatografice HPLC și spectrofotometrice UV-VIS cu scopul de a corela proprietățile analitice cu afinitatea 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 componente de diazotare și ale componente 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 marcarea secțiunilor histologice. Secțiunile sistemului nervos central umane marcate cu acest colorant au pus în evidență toate elementele sale structurale.

Testele de colorare a secțiunilor histologice au fost prelucrate în laboratorul de citohistologie și biologie celulară al Facultății de Medicină Generală.

Keywords: azo-dye; histological dye; staining; biological marker

Introduction

The purpose of this project is the study of the new azo dye 5-[4-hydroxy-3-[[4-(thiazol-2-yl sulfamoyl)phenyl] carbamoyl] phenyl] azo-2-[2-[4-[4-hydroxy-3-[[4-(thiazol-2-ylsulfamoyl) phenyl] carbamoyl] phenyl] azo-2-sulfo-phenyl]vinyl]benzenesulfonic acid (called SINT 7) and the evaluation of its staining properties. The synthesis was conducted using the 4,4'-diaminostilbene-2,2'-disulfonic acid as azo-component and 2-hydroxy-N-[4-(thiazol-2-yl sulfamoyl) phenyl] benzamide, as coupling component. The structure of the 4,4'-diaminostilbene-2,2'-disulfonic acid allows the presence of two isomers: vinyl Z- and E-isomers (Figure 1). The derived azo dye also has two isomers.

Materials and methods

The fundamental analytical study of diazo dye 5-[4-hydroxy-3-[[4-(thiazol-2-yl sulfamoyl)phenyl] carbamoyl] phenyl] azo-2-[2-[4-[4-hydroxy-3-[[4-(thiazol-2-ylsulfamoyl) phenyl] carbamoyl] phenyl] azo-2-sulfo-phenyl]vinyl]benzenesulfonic acid (SINT 7) followed two directions: HPLC and spectrophotometric UV/VIS analysis.

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 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 10-60mg/L (ppm) were prepared.

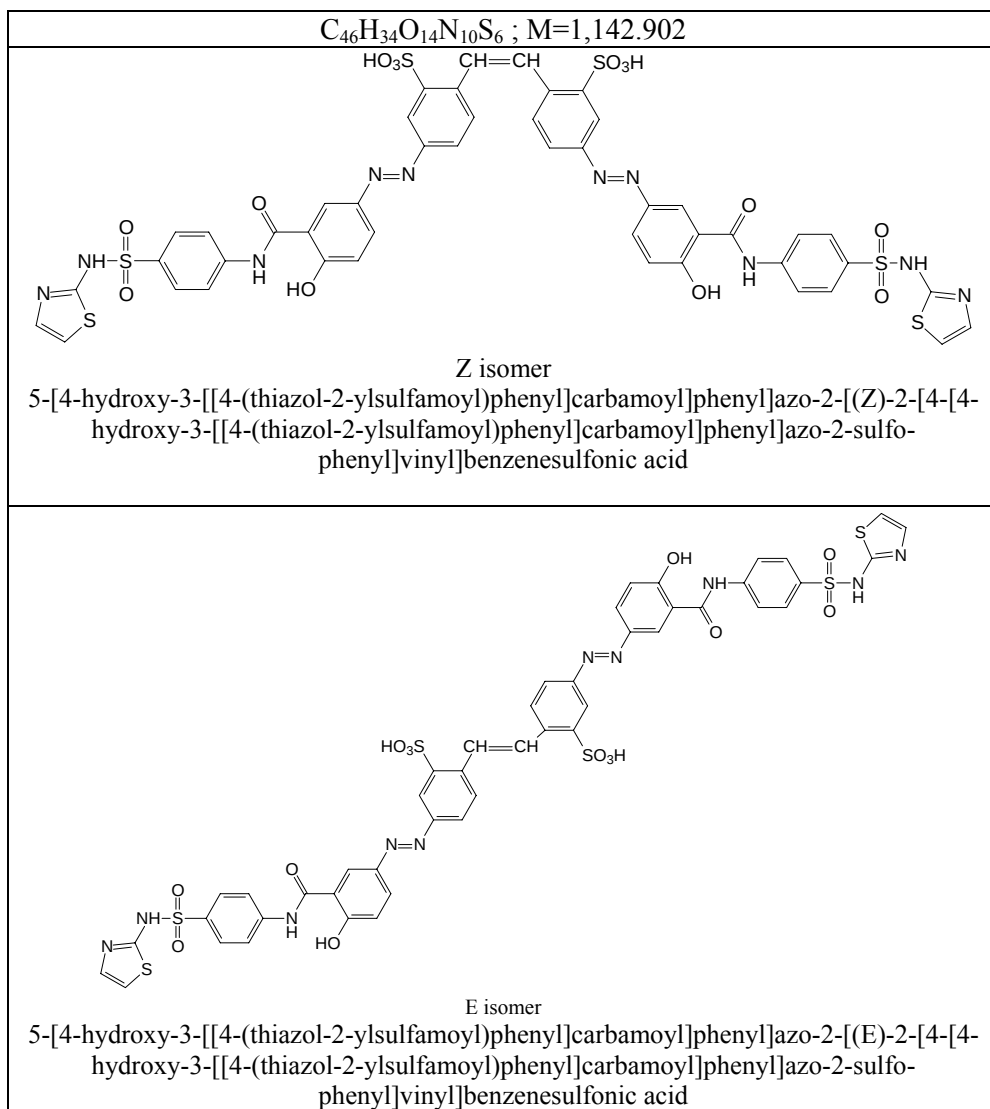


Figure 1

Z and E isomers of SINT 7

For optimum separation, 12 chromatographic methods were tested. The method with the best result was applied in the diazo dye 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 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 (PDA) Setup. The Similarity Index is a number between 0 and 1.

Spectrometric analysis is performed using CINTRA 10e GBC Spectrometer: Wavelength Accuracy $\pm 0.2\text{nm}$; Wavelength Reproducibility $\pm 0.04\text{nm}$; Photometric Accuracy $\pm 0.002\text{A}$; Photometric Repeatability $\pm 0.0001\text{A}$; Photometric Linearity Better 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 100ppm, reveal the wavelength of maximum absorbance and was correlated with the result of chromatographic analysis.

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

Staining method

Central nervous system sections were stained using modified hematoxylin-eosin technique: the eosin was replaced by the new azo dyes. An optical microscope NIKON with video camera JVC connected to computer was used to study each glass slide. The picture of each slide is displayed and commented upon in this article.

Results and discussion

Chromatographic analysis

Standard solution SINT7 (C=84 ppm) in methanol 50% was analysed chromatographically (Sint4.met): Column RP C₁₈ 5 μ 150x4.6 mm, Guard column RP C₁₈ 5 μ , 50x4.6 mm; Mobile phase Methanol/water/sodium pentansulfonat=50/25/25 (v/v/v); Flow rate 1 mL/min; Temperature 30°C; Detector PDA (200-700 nm); units: mAU; Sample size 100 μ L; Sample conc. 84 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: first major 56.2% and the second minor 41.8%, very well separated (resolution 1.095) (Figure 2).

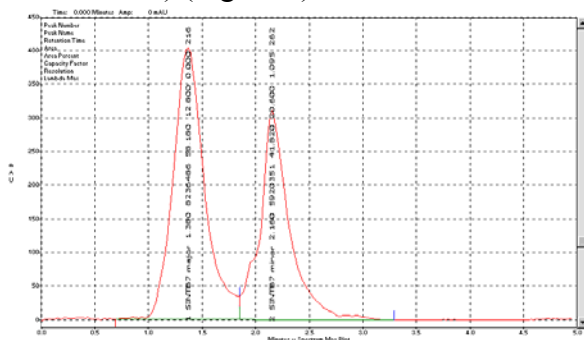
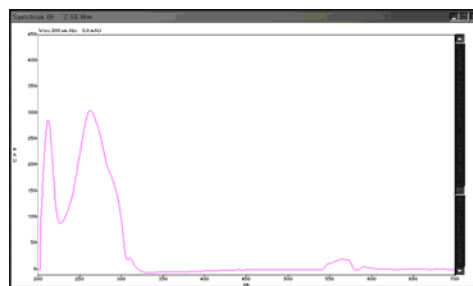
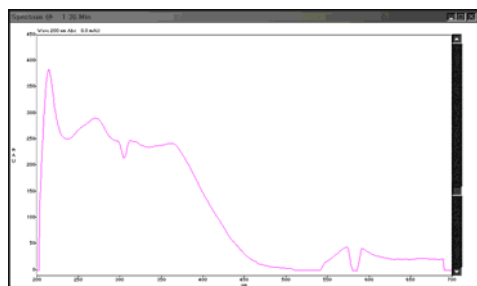


Figure 2

Spectrum Max Plot standard solution SINT 7

The spectral profiles of the two components are quite different: the major component evidences a wide absorption band (320-375 nm) with the maximum of absorbance at 216nm and 275nm, whereas the minor component has two maximum levels of absorbance at 210nm and 262nm. This difference allows the major component analysis in the range of 300-400nm, the minor component manifesting unelectable or no absorbance (Fig. 3).



SINT 7 Spectral profiles of the I-st peak -major

SINT 7 Spectral profiles of the II-nd peak -minor

Figure 3

Spectral profiles of the two SINT 7 components

Overlaid normalized spectra of the two peaks of SINT 7 and spectral profiles of the 4,4'-diaminostilbene-2,2'-disulfonic acid (AASS) and, on the other hand, spectral profiles of the 2-hydroxy-N-[4-(thiazol-2-yl sulfamoyl) phenyl] benzamide turn out the absence of the two precursors in the investigated diazo dye (Fig. 4).

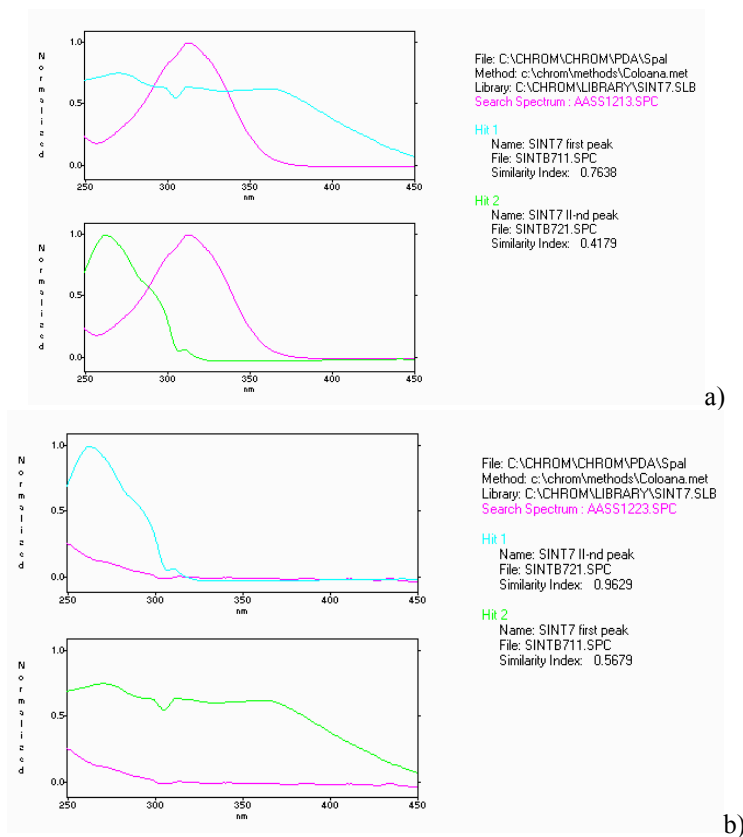


Figure 4

Overlaid normalized spectra of the two peaks of SINT 7 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 7 solutions, in methanol 50%, with the same pH and concentration 10-75ppm, exhibit maximum absorbances in near UV region and an absorption band in VIS (Fig. 5).

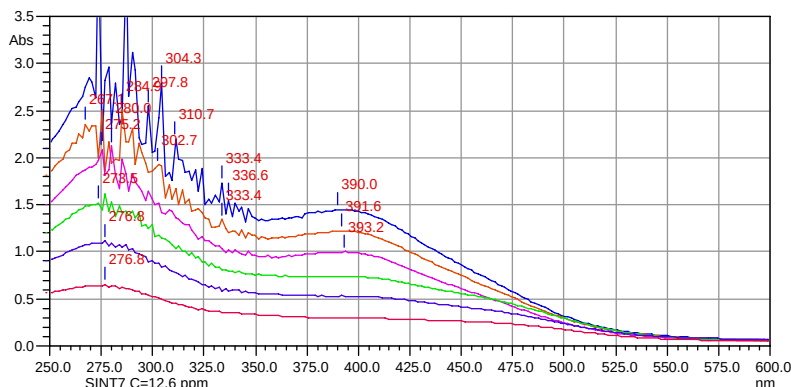


Figure 5

Absorption spectra of standard SINT 7 solutions
in the range of 250 -600nm (C=12-76 ppm)

Spectrophotometric quantitative analysis

Calibration curves of standard SINT 7 solutions (C=12-76 ppm) demonstrate a very good correlation coefficient in all four surveyed wavelengths: 400, 392, 333 and 300nm, according to Lambert-Beer law.

$$C_{\text{SINT 7}} = 54.45 \times A_{400\text{nm}} - 3.529; r = 0.999685$$

$$C_{\text{SINT 7}} = 53.78 \times A_{392\text{nm}} - 3.082; r = 0.999599$$

$$C_{\text{SINT 7}} = 38.08 \times A_{300\text{nm}} - 8.483; r = 0.999424$$

$$C_{\text{SINT 7}} = 54.52 \times A_{333\text{nm}} - 6.779; r = 0.999582$$

In VIS region, only major component exhibit absorbance (Figure 2 and 3), enabling its spectral properties determination.

Spectral parameter computation at 333nm:

$$\text{Calibration curve equation: } A_{333\text{nm}} = 0.0202 \times C_{\text{SINT 7}}$$

$$\text{Absorbivity: } a = 0.0202 \frac{L}{\text{mg} \times \text{cm}} = 20.2 \frac{L}{\text{g} \times \text{cm}}$$

$$\text{Specific absorbance: } A_{1\text{cm}}^{1\%} = 10 \times a = 202$$

$$\text{Molar absorbivity: } \varepsilon = a \times M = 20.2 \times 1,142.9 = 23,086.6 \frac{L}{\text{mol} \times \text{cm}}$$

Spectrophotometric determination of ionization constants

In order to determine ionization constants, 32 standard solutions SINT 7 of the same analytical concentration (C=33.6 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, four dissociation steps were identified [3]:
 In strong acidic medium pH=0.7-1.3, isosbestic point at $\lambda=352$ and 430 nm, $pK_{a,1}=1$.
 In acidic medium pH= 1-2.6, isosbestic point at $\lambda=335$ nm, $pK_{a,2}=1.8$.
 In cvasineutral medium pH= 4-5.5, isosbestic point at $\lambda=423.7$ nm, $pK_{a,3}=5$.
 In alkaline medium pH= 9-12, isosbestic point at $\lambda=394$ nm, $pK_{a,4}=11$.

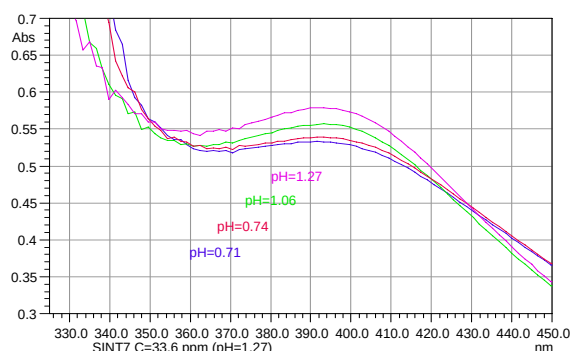


Figure 6

Absorption spectra of standard SINT 7 solutions of the same analytical concentration (33.6 mg/L), in buffer solutions (pH= 0.7-1.3) in the range 320-450nm

Central nervous system staining

5-[4-hydroxy-3-[[4-(thiazol-2-yl sulfamoyl)phenyl] carbamoyl] phenyl] azo-2-[2-[4-[4-hydroxy-3-[[4-(thiazol-2-ylsulfamoyl) phenyl] carbamoyl] phenyl] azo-2-sulfo-phenyl]vinyl]benzenesulfonic acid (SINT 7) biological marker.

Human cerebral cortex staining with SINT 7 (Fig. 1) revealed pyramidal cells, with violet nucleus, granular neurons with blue-violet nucleus, blood vessels and glial cells. The astrocytes are star-shaped cells with processes closely applied to capillar blood vessels. Their cytoplasm contains glial filaments made up of glial fibrillar acidic protein (GFAP). Only special histochemical stains for GFAP help identify astrocytes in tissue sections.

The region between pyramidal and glial cells is termed the *neuropil* and is filled with glial and nerve cell.

Figure 2 represents the image of human cerebral cortex stained with usual hematoxylin-eosin technique. The advantages of synthetic dye are very clear when comparing the two images.

Staining with synthetic dye cerebellum cortex (fig. 3) showed neurons from the molecular layer, Purkinje cells and granular cells (neurons of 3rd layer). Neurons nuclei appear are very easily identified. Purkinje cells

appear in red-brown with brown-black nucleolus. Granular neurons appear in blue. Dendrites and axons are stained in brown with a good resolution. The background is yellow-brown. Discrimination between the three layers of cerebellum cortex is very good. The surveyed exhibit with the new azo dyes is capable of demonstrating, in a single preparation, all the components of the cerebellum cortex section.

Figure 4 represents the image of human spinal cord staining with SINT7.

Perikaryon: It is multipolar (i.e., possesses a single axon and several dendrites) and features a proeminent central nucleus. The cytoplasm is rich in Nissl bodies except in the axon hillock (lighter area of cell body the axon arises from).

Dendrites: Stout tapering processes similar in structure to the perikaryon.

Axon: This arises from the axon hillock. It is a slender process of uniform diameter and great length. The myelin sheath is not seen around the axon in this preparation because it is not preserved by the fixation method used (10% formalin).

Dye solution used to stain biological sections is 0.25% in methanol and has a pH=4. At this pH, the dye is between the second and third dissociation step. Ionization form enables binding to proteins with negative and positive charges. This may be the explanation for the different colours given to each component of the structure.

The staining of human central nervous system sections

Human cerebral cortex sections (Ocular 10x, ob.10x)

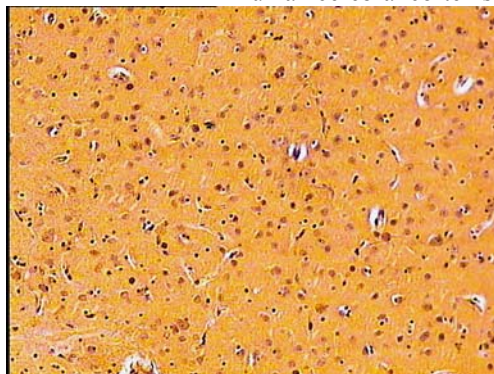


Figure 1

Human cerebral cortex staining with SINT 7

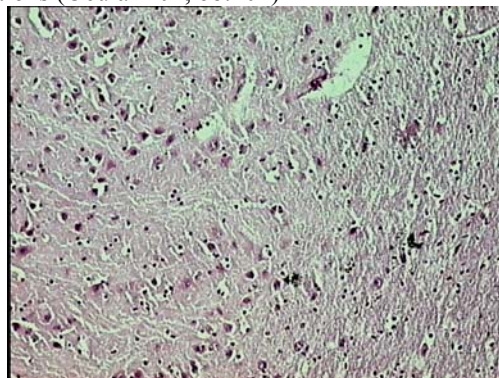
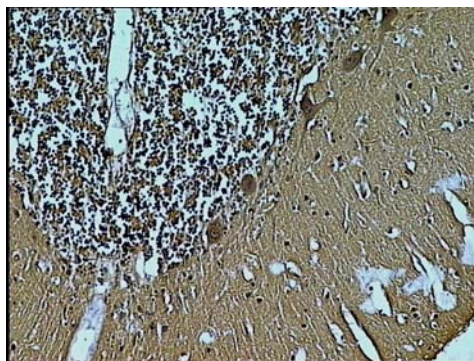
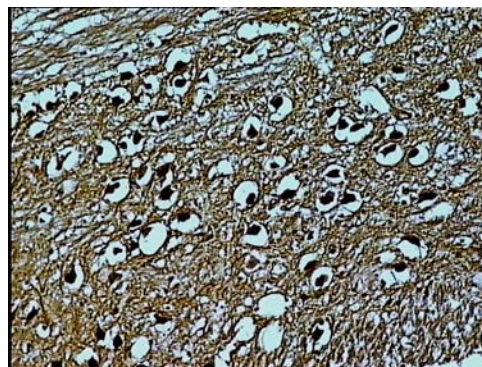


Figure 2

Human cerebral cortex staining with
Hematoxylin-Eosin

**Figure 3**

Human cerebellum cortex staining with SINT 7 (Ocular 10x, Ob.10x)

**Figure 4**

Human spinal cord staining with SINT 7 (Ocular 10x, Ob.10x)

Conclusions

The new diazoic dye obtained by synthesis, 5-[4-hydroxy-3-[[4-(thiazol-2-yl sulfamoyl)phenyl] carbamoyl] phenyl] azo-2-[2-[4-[4-hydroxy-3-[[4-(thiazol-2-ylsulfamoyl) phenyl] carbamoyl] phenyl] azo-2-sulfo-phenyl]vinyl]benzenesulfonic acid (SINT 7) was analyzed by HPLC and spectrophotometric methods and tested for biological marker applications.

The HPLC methods revealed the presence of two components and the absence of synthesis precursors. The major component displays a large absorption band in VIS region and the minor one only in UV. Since conjugation of chromophores and auxochromes is better in E isomer, absorbance shifts to longer 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 kept abreast two directions: spectral parameters computation (absorbivity, specific absorbance and molar absorbivity) in near VIS region, meant for E isomer and ionization constants determination for the four dissociation steps.

Staining method

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

The Golgi silver methods selectively impregnate relatively few cells, but they accomplish this most completely. These methods are useful in

outlining the external shape of nerve cells and their processes (especially dendrites) but do not reveal details of internal cell structure [6].

The new azo dye was studied as a biological dye for central nervous system. It provided 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 techniques. The methods date from the beginning of XXth century. The new method is simpler, the reagents are accessible and the results are very good. The staining procedure was carried through in the Cytodiagnostic Laboratory of the "Ovidius" University of Constanta Faculty of Medicine, with the professional support of prof. Teofil Mehedinti, MD, PhD. The results were compared with customary staining methods using silver and gold salts and hematoxylin-eosin reagent.

Acknowledgments

Reverential postmortem gratitude to Prof. Dan Ulmeanu, MD, PhD who regrettably past away recently. His authoritative scientific competence contributed to making this report possible.

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