Synthesis and Characterization of Novel Color Chemosensors Based on Azo Dyes for Possible Application in Opioid Pharmacology

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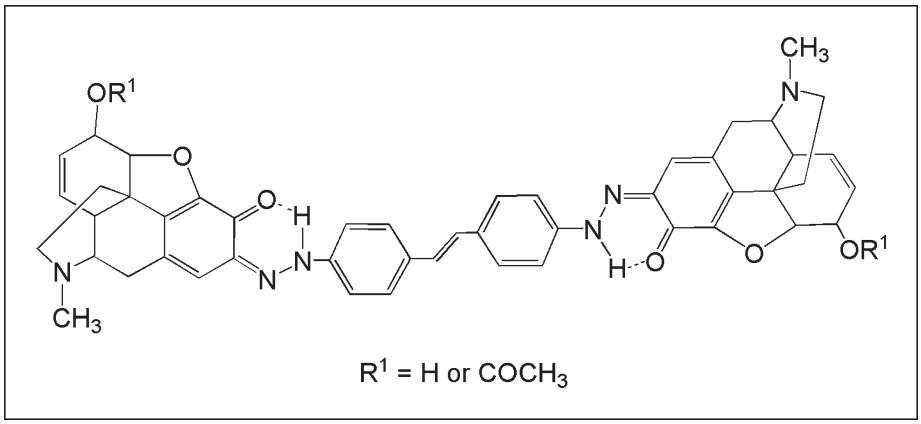

An applicable strategy of chemical labeling of morphine (M) and 6-acetyl morphine (6-AM) using diazonium salts is described. M or 6-AM was coupled with aryl diazoinum salts to give morphine azo dyes. The coupling of the diazotized 4,4′-diaminostilbene with M or 6-AM in ratio 1:2 gave stilbene-based azo dyes containing two M or 6-AM units, respectively. Diazotization of 5-(*p*-aminophenyl)-10,15,20-triphenylporphyrin and subsequent azo coupling of the diazoniun salt with M and with 6-AM was our route to get highly fluorescent morphine dyes. The resulting dyes can exist in two possible tautomeric structures, azo and hydrazone, stabilized to a significant extent by intramolecular H-bonding. It was shown that these dyes exists predominantly or exclusively in their hydrazone form. This conclusion is drawn from the lack of a distinct band in the 380–420 nm region of the absorption spectra (a region in which the long wavelength absorption band for the azo-form is observed), together with results of NMR studies in deuterated DMSO. The tautomeric properties of the compounds were judged by density functional calculations at the B3LYP/6-31G* and B3LYP/6-31G** levels. The analysis of spiked compounds in human urine samples was studied by capillary electrophoresis (CE) with UV-fluorescence photo-diode array detectors.

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INTRODUCTION

A great amount of attention continues to be devoted to the development of synthetic molecular receptors with the ability to recognize neutral organic species, including abused drugs. The morphine alkaloids comprise a family of structurally related natural products of unique clinical importance in medicine [1]. Morphine is a fascinating compound that has been used as an efficient analgesic and is indispensable in treating pains associated with cancer [2]. Morphine (M) is also found in normal brain, blood, and liver tissue [3]. However, it is strictly controlled by authorities due to its addictive nature. On the other hand, the unusual architecture of M has offered a continuing challenge to the art and science of organic synthesis (Fig. 1) [4,5]. Hence, a number of morphine derivatives have been reported to date [6]. Heroin, which is obtained synthetically from the acetylation of M, has an analgesic potency two to three times that of the parent drug and, due to the two acetyl groups, has better penetration across the blood-brain barrier [7]. Heroin itself is rarely present in detectable quantities in body fluids. The drug hydrolyses rapidly to 6-acetylmorphine (6-AM), which in turn hydrolyses to M. Therefore, heroin consumption can be confirmed by identifying its two primary metabolites [8,9]. In addition, heroin is different from most other opioids in that it has little or no affinity for opioid receptors in the brain. The analgesic effects of the drug are attributed to the combined effect of 6-AM and M [2].

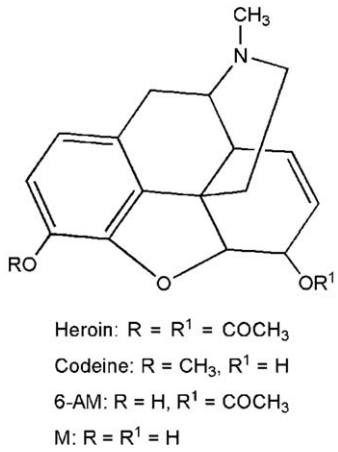


Figure 1. Schematic structures of some abused drugs [heroin, codeine, 6-acetylmorphine (6-AM), and morphine (M)].

It is generally accepted that two sites, the basic nitrogen and the phenol moiety, are necessary for analgesic binding to its receptors [10,11]. The phenolic hydroxyl group is recognized as a requisite for the formation of a hydrogen bond with a dipolar site on the receptor and for good antinociceptive activity [11,12]. However, the free hydroxyl group is also a potential site for metabolism, conjugation, and excretion resulting in low oral bioavailability and short duration of action [13,14]. One of the approaches to improve the pharmacological properties of analogues is to modify this phenolic hydroxyl function. Several potent compounds have been synthesized and identified by replacing the hydroxyl moiety of morphinans with other functional groups (amino, carboxamido, 2-aminothiazole) [15,16].

The use of dyes in chemistry, biology, and medicine is growing continuously, with many new applications in the diagnosis and treatment of disease [17–19]. Moreover, azo dyes have been known for over forty years, where they were used for investigations in cancer treatment [20]. Numerous fluorescent probes for monosaccharides based on azo dyes have been described in the literature. Moreover, many abused drugs (*i.e.*, heroin, codine, 6-AM and M) are tertiary amines (Fig. 1) and are not compatible with the most commonly utilized amine reactive fluorescent dyes. These dyes include compounds such as fluorescein isothiocyanate isomer I (FITC) [21], 4-(4,6-dichloro-*s*-triazin-2-ylamino)fluorescein (DTAF) [22], 4-fluoro-7-nitrobenzofurazan (NBD-F) [23], and 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) [24]. Other fluorogenic reagents specifically made for derivatization of the tertiary amine group such as the malonic acid/acetic anhydride system [25] and the aconitic acid method [26] result in a deteriorating effect on the fluorescence of the reaction product. In addition, the products of these reactions are unstable, light sensitive, and give many components that seem to be associated with the reagent blank.

The most important problems for development of a new morphine detector are tedious and time-consuming reaction steps. In an effort to develop novel morphine derivatives that are effective as chemosensors for heroin use at very low concentrations, herein we report fast, economic, and simple approaches to the synthesis of a novel series of highly fluorescence azo-morphine dyes. Compared with the previously reported methods [20–27], the present test produces an intense color which is not affected by the presence of any diluents or adulterants and which is easily adapted to field use. Diaminostilbene and porphyrin related dyes are strongly light absorbing and highly luminescent [28,29]. These dyes are covalently attached to proteins and other biological and nonbiological materials to make these materials fluorescent so that they can be detected. The binding advantage of *trans*-4,4′-bis-diazostilbene or diazoporphyrin over diazobenzene is production of highly fluorescent dyes that can be detected even at very low morphine concentration. The developed method was used for determination of highly diluted M and 6-AM in spiked human urine sample with very high accuracy and precision.

EXPERIMENTAL

Materials. All chemicals and reagents were commercially available and were used as received. Most of solvents were at least of reagent grade and were used without further purification. M, 6-AM, and codeine were obtained from Lipomed Inc. (One Broaway, Cambridge, MA, USA). 5-(*p*-Aminophenyl)-10,15,20-triphenylporphyrin was prepared according to the literature [30]. Analytical thin layer chromatography (TLC) was performed on a glass plates of silica gel 60 GF_{254} (Merck). Visualization was accompanied by UV light (254 nm). Column chromatography was conducted on silica gel 60 (Merck 70–230 mesh). ^{1}H NMR and ^{13}C NMR spectra were measured on JEOL JNM-AL 300 (300 MHz) and VARIAN UNITY-INOVA 400 (400 MHz) spectrometers. Chemical shifts of ^{1}H NMR and ^{13}C NMR were expressed in parts per million (ppm, δ units), and coupling constant was expressed in units of Hertz (Hz). Elemental analyses were performed by a Perkin-Elmer 2400 automatic elemental analyzer. All compounds gave elemental analysis within ±0.4%. Electrospray ionization (ESI) mass spectra were recorded on a Shimadzu LCMS-2010 eV spectrometer in CH_3OH. The UV–vis data were measured on Shimadzu 3101 PC instrument. The fluorescence (excitation and emission) spectra were determined with Perkin Elmer Lambada 50 PC spectrophotometer: excitation slit width = 5 nm, emission slit width = 5 nm. AP/ACE MDQ CE system coupled with photo-diode array detectors (PAD) supplied from Beckman (Fullerton, CA, USA) was used throughout the experiments. Separation was carried out in a 50.2 cm long × 50 μm (10 cm to the detector, short way). After each

experiment, the capillary was washed with 0.1 mol dm^{-3} sodium hydroxide for 2.0 min, distilled water for 1.0 min, and the separation electrolyte for 2.0 min. Hydrodynamic injection mode was applied for sample loading. 32 Karat version 7.0 supplied from Beckman (Fullerton, CA, USA) was used for controlling the CE system as well as data acquisition and processing.

General procedure for the synthesis of compounds 1–6. ***Diazotization.*** A 0–5°C solution of substituted aniline (0.5 mmol) and 1*N* HCl (2 mL) in deionized (DI) water (5 mL) was treated with a 0–5°C solution of $NaNO_2$ (100 mg, 1.5 mmol) in DI water (5 mL) and the diazotization continued for 10 min.

Coupling. The resulting diazonium salt solution was poured into a 0–5°C solution of M or 6-AM (0.5 mmol) in NaOH (50 mg, 1.25 mmol, 5 mL). The mixture was stirred at 0–5°C for another 10 min. The resulting precipitate was filtered off, washed with NaCl, DI water, and dried *in vacuo*. The products were purified by flash column chromatography using hexane and ethylacetate in ratio 2:1 as eluent. Upon storage of the azo coupling products **1–6** at ambient temperature for several months neither change in their UV–vis spectra nor appearance of foreign signals in their ^{1}HNMR spectra were observed, which provides evidence of their stability.

4-(Morphine-2-yl-azo)benzenesulfonic acid (1). Yield: 200 mg (87%), mp = 156–158°C, R_f = 0.48 as a red solid; IR (KBr) ν: 3385, 3379 (OH), 1641 (C=C, alkene), 1620 (C=O, hydrazone), 1605 (C=N), 1525 (C=C, aromatic), 1350, 1150 (SO_2), 1282, 1091 (C—O—C) cm^{-1}; ^{1}H NMR (300 MHz, DMSO): δ = 12.75 (br s, 1H, NHO), 7.78 (d, *J* = 7.85 Hz, 2H, aromatic H), 7.56 (d, *J* = 7.85 Hz, 2H, aromatic H), 7.02 (s, 1H, aromatic H), 5.53 (d, 1H, *J* = 9.12 Hz, CH=CH), 5.28 (d, 1H, *J* = 9.12 Hz, CH=CH), 4.76 (d, 1H, *J* = 7.5 Hz), 4.23–4.27 (m, 1H), 3.37–3.32 (m, 1H), 3.01 (d, 1H, *J* = 17.5 Hz), 2.59–2.64 (m, 1H), 2.56 (d, 1H, *J* = 7.05 Hz), 2.42 (dd, 1H, *J* = 5.24 Hz), 2.36 (s, 3H, NCH_3), 2.25 (dd, 1H, *J* = 4.2 Hz), 1.96 (td, 1H, *J* = 9.32 Hz, *J* = 5.12 Hz), 1.87 (d, 1H, *J* = 10.54 Hz); ^{13}C NMR (75 MHz, DMSO) δ = 183.23 (C=O), 156.59, 153.45, 147.23, 139.75, 131.17, 126.5, 42.95 (C), 133.05, 129.89, 128.12, 125.15, 118.23, 117.76, 91.25, 68.45, 59.34, 40.86 (CH), 45.56, 35.05, 21.06 (CH_2), 42.69 (N—CH_3); MS (ESI), *m*/*z*(%): 469 (100) [M^+]; Anal. Calcd. for $C_{23}H_{23}N_3O_6S$ (469.51): C 58.84, H 4.94, N 8.95; found: C 58.81, H 4.89, N 8.92.

2-(m-Carboxy-phenylazo)morphine (2). Yield: 165 mg (76%), mp = 184–186°C, R_f = 0.32 as an orange solid; IR (KBr) ν: 3382, 3375 (OH), 1712 (C=O, carboxylic), 1642 (C=C, alkene), 1618 (C=O, hydrazone), 1595 (C=N), 1521 (C=C, aromatic), 1280, 1087 (C—O—C) cm^{-1}; ^{1}H NMR (300 MHz, DMSO): δ = 13.25 (br s, 1H, NHO), 7.86–7.45 (m, 4H, aromatic H), 6.85 (s, 1H, aromatic H), 5.57 (d, 1H, *J* = 9.0 Hz, CH=CH), 5.32 (d, 1H, *J* = 9.0 Hz, CH=CH), 4.69 (d, 1H, *J* = 7.42 Hz), 4.2–4.25 (m, 1H), 3.35–3.3 (m, 1H), 3.04 (d, 1H, *J* = 15.9 Hz), 2.54–2.59 (m, 1H), 2.51 (d, 1H, *J* = 7.0 Hz), 2.43 (dd, 1H, *J* = 4.86 Hz), 2.35 (s, 3H, NCH_3), 2.25 (dd, 1H, *J* = 4.12 Hz), 1.97 (td, 1H, *J* = 9.67 Hz, *J* = 5.52 Hz), 1.91 (d, 1H, *J* = 10.05 Hz); ^{13}C NMR (75 MHz, DMSO) δ = 181.83, 177.56 (C=O), 155.69, 145.36, 138.45, 131.65, 126.85, 42.64, (C), 133.21, 126.37, 126.05, 125.87, 125.12, 124.86, 119.45, 118.69, 91.86, 67.75, 58.94, 40.25 (CH), 45.12, 34.68, 21.46 (CH_2), 42.85 (N—CH_3); (ESI): *m*/*z*(%): 433 (85) [M^+]; Anal. Calcd. for $C_{24}H_{23}N_3O_s$ (433.46): C 66.50, H 5.35, N 9.69; found: C 66.42, H 5.29, N 9.61.

2-(p-Methoxy-phenylazo)morphine (3). Yield: 193 mg (92%), mp = 167–169°C, R_f = 0.39 as red solid; IR (KBr) ν: 3382, 3375 (OH), 1635 (C=C, alkene), 1624 (C=O, hydrazone), 1610 (C=N), 1523 (C=C, aromatic), 1287, 1094 (C—O—C) cm^{-1}; ^{1}HNMR (300 MHz, DMSO): δ = 12.75 (br s, 1H, NHO), 7.79 (d, *J* = 8.12 Hz, 2H, aromatic H), 7.54 (d, *J* = 8.12 Hz, 2H, aromatic H), 6.97 (s, 1H, aromatic H), 5.42 (d, 1H, *J* = 9.25 Hz, CH=CH), 5.25 (d, 1H, *J* = 9.25 Hz, CH=CH), 4.68 (d, 1H, *J* = 7.51 Hz), 4.22–4.25 (m, 1H), 3.41–3.55 (m, 1H), 3.02 (d, 1H, *J* = 17.32 Hz), 2.58–2.65 (m, 1H), 2.57 (d, 1H, *J* = 7.19 Hz), 2.45 (dd, 1H, *J* = 5.35 Hz), 2.36 (s, 3H, NCH_3), 2.27 (dd, 1H, *J* = 4.17 Hz), 2.05 (td, 1H, *J* = 9.05 Hz, *J* = 5.6 Hz), 1.92 (d, 1H, *J* = 10.51 Hz); ^{13}CNMR (75 MHz, DMSO) δ = 183.52 (C=O), 158.45, 156.79, 146.45, 138.95, 131.57, 126.87, 42.45, (C), 132.98, 128.45, 127.96, 125.34, 119.12, 118.07, 91.35, 67.85, 58.96, 40.84 (CH), 45.56, 35.05, 21.06 (CH_2), 60.13 (O—CH_3), 42.69 (N—CH_3). (ESI): *m*/*z*(%) 419 (93) [M^+]; Anal. Calcd. for $C_{24}H_{25}N_3O_4$ (419.47): C 68.72, H 6.01, N 10.02; Found: C 68.67, H 6.00, N 10.00.

2-(p-Nitro-phenylazo)morphine (4). Yield: 182 mg (84%), mp = 192–194°C, R_f = 0.41 as a red solid. IR (KBr) ν: 3380, 3375 (OH), 1638 (C=C, alkene), 1620 (C=O, hydrazone), 1600 (C=N), 1520 (C=C, aromatic), 1517, 1334, (NO_2), 1282, 1091 (C—O—C) cm^{-1}; ^{1}HNMR (300 MHz, DMSO): δ = 13.52 (br s, 1H, NHO), 7.82 (d, *J* = 6.52 Hz, 2H, aromatic H), 7.65 (d, *J* = 6.52 Hz, 2H, aromatic H), 7.05 (s, 1H, aromatic H), 5.62 (d, 1H, *J* = 9.07 Hz, CH=CH), 5.19 (d, 1H, *J* = 9.07 Hz, CH=CH), 4.72 (d, 1H, *J* = 6.95 Hz), 4.37–4.32 (m, 1H), 3.35–3.29 (m, 1H), 3.0 (d, 1H, *J* = 16.98 Hz), 2.55–2.6 (m, 1H), 2.52 (d, 1H, *J* = 7.0 Hz), 2.45 (dd, 1H, *J* = 5.1 Hz), 2.35 (s, 3H, NCH_3), 2.24 (dd, 1H, *J* = 4.56 Hz), 1.94 (td, 1H, *J* = 8.79 Hz, *J* = 5.12 Hz), 1.85 (d, 1H, *J* = 10.44 Hz); ^{13}CNMR (75 MHz, DMSO) δ = 182.54 (C=O), 157.55, 155.05, 147.15, 139.05, 132.02, 126.52, 42.63, (C), 132.85, 128.49, 128.02, 125.65, 119.22, 118.47, 91.05, 67.75, 58.72, 40.79 (CH), 45.34, 34.79, 22.12 (CH_2), 42.77 (N—CH_3). (ESI): *m*/*z*(%) 434 (91) [M^+]; Anal. Calcd. for $C_{23}H_{22}N_4O_s$ (434.44): C 63.59, H 5.10, N 12.90; found: C 63.54, H 5.06, N 12.86.

4-(6-Acetylmorphine-2-yl-azo)benzenesulfonic acid (5). Yield: 230 mg (90%), mp = 145–147°C, R_f = 0.5 as a red solid; IR (KBr) ν: 3382, 3375 (OH), 1722 (C=O, acetyl), 1640 (C=C, alkene), 1619 (C=O, hydrazone), 1602 (C=N), 1521 (C=C, aromatic), 1350, 1150 (SO_2), 1285, 1095 (C—O—C) cm^{-1}; ^{1}HNMR (300 MHz, DMSO): δ = 13.27 (br s, 1H, NHO), 7.77 (d, *J* = 7.9 Hz, 2H, aromatic H), 7.58 (d, *J* = 7.9 Hz, 2H, aromatic H), 7.0 (s, 1H, aromatic H), 5.52 (d, 1H, *J* = 9.02 Hz, CH=CH), 5.25 (d, 1H, *J* = 9.02 Hz, CH=CH), 4.75 (d, 1H, *J* = 7.32 Hz), 4.25–4.29 (m, 1H), 3.35–3.4 (m, 1H), 3.04 (d, 1H, *J* = 17.56 Hz), 2.59–2.64 (m, 1H), 2.53 (d, 1H, *J* = 7.0 Hz), 2.40 (dd, 1H, *J* = 5.24 Hz), 2.35 (s, 3H, NCH_3), 2.26 (dd, 1H, *J* = 4.12 Hz), 2.15 (s, 3H, $COCH_3$), 2.0 (td, 1H, *J* = 8.75 Hz, *J* = 5.25 Hz), 1.9 (d, 1H, *J* = 10.26 Hz); ^{13}CNMR (75 MHz, DMSO) δ = 181.76, 172.56 (C=O), 157.21, 154.24, 147.05, 138.95, 131.55, 126.65, 42.31, (C), 132.46, 129.44, 128.75, 125.19, 118.25, 117.86, 91.25, 68.45, 59.34, 40.86 (CH), 45.56, 35.05, 22.06 (CH_2), 42.69, 21.17 (N—CH_3, $COCH_3$). (ESI): *m*/*z*(%) 511 (96) [M^+]; Anal. Calcd. for

$C_{25}H_{25}N_3O_7S$ (511.55): C 58.7, H 4.93, N, 8.21; found: C 58.57; H, 4.91; N, 8.15.

2-(m-Carboxy-phenylazo)-6-acetylmorphine (6). Yield: 202 mg (85%), mp = 177–179°C, R_f = 0.35 as an orange solid; IR (KBr) ν: 3377 (OH), 1725 (C=O, acetyl), 1718 (C=O, carboxylic), 1639 (C=C, alkene), 1619 (C=O, hydrazone), 1592 (C=N), 1525 (C=C, aromatic), 1282, 1085 (C—O—C) cm^{-1}; ^{1}HNMR (300 MHz, DMSO): δ = 13.39 (br s, 1H, NHO), 7.82–7.5 (m, 4H, aromatic H), 6.89 (s, 1H, aromatic H), 5.55 (d, 1H, *J* = 8.7 Hz, CH=CH), 5.34 (d, 1H, *J* = 8.7 Hz, CH=CH), 4.7 (d, 1H, *J* = 7.75 Hz), 4.24–4.27 (m, 1H), 3.36–3.32 (m, 1H), 3.03 (d, 1H, *J* = 15.35 Hz), 2.55–2.61 (m, 1H), 2.51 (d, 1H, *J* = 7.4 Hz), 2.45 (dd, 1H, *J* = 4.85 Hz), 2.3 (s, 3H, NCH_3), 2.25 (dd, 1H, *J* = 4.6 Hz), 2.09 (s, 3H, CH_3), 1.93 (td, 1H, *J* = 9.11 Hz, *J* = 5.25 Hz), 1.89 (d, 1H, *J* = 10.29 Hz); ^{13}CNMR (75 MHz, DMSO) δ = 185.05, 176.98, 172.89 (C=O), 155.43, 145.35, 138.42, 131.62, 126.25, 42.65, (C), 133.27, 126.57, 126.11, 125.23, 125.34, 124.67, 119.49, 118.21, 91.28, 67.45, 58.75, 40.36 (CH), 45.16, 34.59, 21.61 (CH_2), 42.85, 21.99 (N—CH_3, $COCH_3$); (ESI): *m*/*z*(%): 475 (88) [M^+]; Anal. Calcd. for $C_{26}H_{25}N_3O_6$ (475.49): C 65.67, H 5.30, N 8.84; found: C 65.51, H 5.29, N 8.79.

General procedure for the synthesis of compounds 7 and 8. These compounds were prepared from M or 6-AM (0.5 mmol) and *trans*, 4,4′-diaminostilbene (407 mg, 1.2 mmol), using the procedure described for **1–6**.

Trans-4,4′-bis(morphine-2-yl-azo)stilbene (7). Yield: 365 mg (91%), mp = 199–201°C, R_f = 0.45 as a red solid; IR (KBr) ν: 3385, 3376 (OH), 1642 (C=C, alkene), 1624 (C=O, hydrazone), 1605 (C=N), 1527 (C=C, aromatic), 1284, 1087 (C—O—C) cm^{-1}; ^{1}HNMR (300 MHz, DMSO): δ = 13.45 (br s, 2H, NHO), 7.80 (d, 2H, aromatic H), 7.54 (t, 2H, aromatic H), 7.44 (s, 2H, CH=CH), 4.55 (m, 2H), 4.21–4.26 (m, 2H), 3.38–3.33 (m, 2H), 3.0 (m, 2H), 2.59–2.64 (m, 1H), 2.55 (m, 2H), 2.39 (m, 2H), 2.32 (s, 6H, NCH_3), 2.21 (m, 2H), 1.99 (m, 2H), 1.89 (m, 2H); ^{13}CNMR (75 MHz, DMSO) δ = 182.47 (C=O), 155.98, 146.75, 138.85, 131.27, 126.35, 42.56, (C), 133.12, 129.59, 128.19, 125.16, 118.29, 117.77, 91.45, 68.86, 59.51, 52.43, 40.86 (CH), 45.52, 35.12, 22.12 (CH_2), 42.47 (N—CH_3); (ESI): *m*/*z*(%) 802 (85) [M^+]; Anal. Calcd. for $C_{48}H_{46}N_6O_6$ (802.92): C 71.80, H 5.77, N 10.47; Found: C 71.76, H 5.73, N 10.44.

Trans-4,4′-bis(6-acetylmorphine-2-yl-azo)stilbene (8). Yield: 365 mg (91%), mp = 212–214°C, R_f = 0.52 as a red solid; IR (KBr) ν: 3384, 3377 (OH), 1730 (C=O, acetyl), 1640 (C=C, alkene), 1625 (C=O, hydrazone), 1602 (C=N), 1522 (C=C, aromatic), 1281, 1085 (C—O—C) cm^{-1}; ^{1}HNMR (300 MHz, DMSO): δ = 13.49 (br s, 2H, NHO), 7.78 (d, 2H, aromatic H), 7.61 (t, 2H, aromatic H), 7.46 (s, 2H, CH=CH), 4.52 (m, 2H), 4.22–4.27 (m, 2H), 3.4–3.36 (m, 2H), 3.01 (m, 2H), 2.62–2.64 (m, 1H), 2.52 (m, 2H), 2.35 (m, 2H), 2.27 (s, 6H, NCH_3), 2.23 (m, 2H), 2.19 (s, 6H, $COCH_3$), 1.97 (m, 2H), 1.86 (m, 2H); ^{13}CNMR (75 MHz, DMSO) δ = 181.94, 171.67 (C=O), 155.43, 147.05, 139.05, 132.05, 126.63, 42.83, (C), 133.24, 129.69, 128.23, 125.17, 118.23, 117.46, 91.69, 68.57, 59.38, 52.46, 40.82 (CH), 45.62, 35.45, 22.61 (CH_2), 42.49 (N—CH_3); (ESI): *m*/*z*(%) 886 (90) [M^+]; Anal. Calcd. for $C_{52}H_{50}N_6O_8$ (886.99): C 70.41, H 5.68, N 9.47; Found: C 70.39, H 5.67, N 9.45.

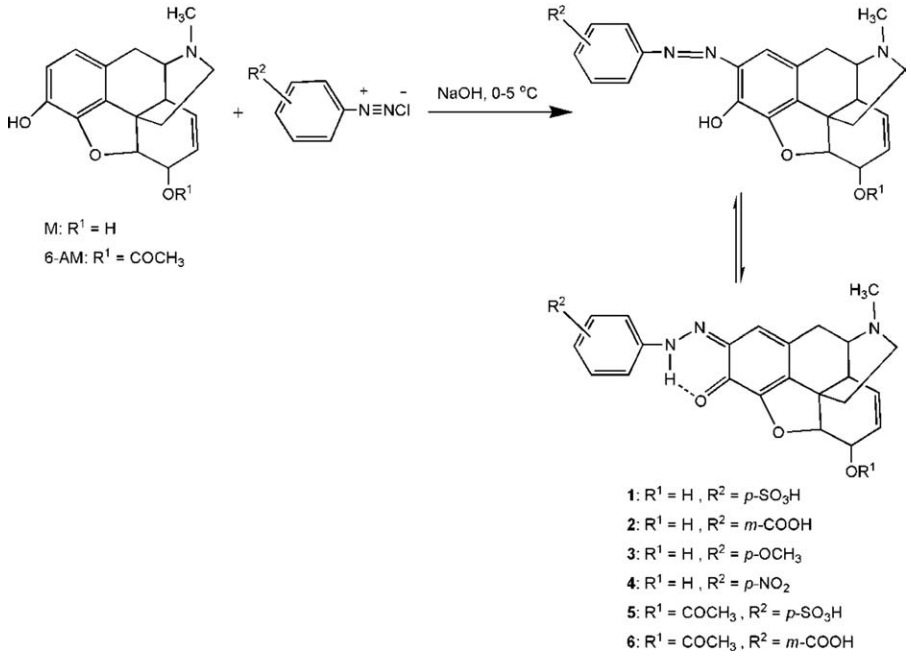
General procedure for the synthesis of compounds 10 and 11. A 0–5°C solution of sodium nitrite (0.12 g, 1.74 mmol) in water (2 mL) was added dropwise to a stirred solution of 5-(*p*-aminophenyl)-10,15,20-triphenylporphyrin (785 mg, 1.25 mmol) in 1*N* HCl (5 mL). The mixture was stirred at 5°C for 15 min. A solution of sodium acetate (0.14 g, 1.71 mmol) in water (5 mL) and M or 6-AM (1.11 mmol) in 3% aqueous NaOH (5 mL) were added to the diazonium salt solution. Then, the reaction mixture was stirred at room temperature for 15 min and diluted to 100 mL with water and filtered. The filtrate was neutralized with HCl to pH 7, the porphyrin filtered off, washed with aqueous ammonia solution (10%), then with water, and dried to constant weight at room temperature. For purification, the porphyrin was dissolved in boiling ether (50 mL) and chromatographed on a column (2.5 cm × 60 cm) of silica gel eluting with ether. The elute was evaporated to 5 mL and porphyrin (**10** or **11**) was precipitated with hexane (20 mL).

5-(Morphine-2-yl-azophenyl)10,15,20-triphenylporphyrin (10). Yield: 950 mg, (82%), mp = 230–232°C, R_f = 0.37 as a voilet solid; IR (KBr) ν: 3384, 3377 (OH), 3310 (CH), 2989, 2927 (NH), 1638 (C=C, alkene), 1625 (C=O, hydrazone), 1604(C=N), 1525 (C=C, aromatic), 1280, 1087 (C—O—C) cm^{-1}; ^{1}HNMR (300 MHz, DMSO): δ = 13.52 (br s, 2H, NHO), 8.65–8.96 (m, 8H, β-pyrrole), 7.02–8.21 (m, 19H, H_{arom}), 5.64 (d, 1H, *J* = 9.05 Hz, CH=CH), 5.2 (d, 1H, *J* = 9.05 Hz, CH=CH), 4.75 (d, 1H, *J* = 7.0 Hz), 4.35–4.25 (m, 1H), 3.35–3.29 (m, 1H), 3.02 (d, 1H, *J* = 17.0 Hz), 2.55–2.6 (m, 1H), 2.52 (d, 1H, *J* = 8.42 Hz), 2.45 (dd, 1H, *J* = 5.5 Hz), 2.35 (s, 3H, NCH_3), 2.25 (dd, 1H, *J* = 4.5 Hz), 1.99 (td, 1H, *J* = 8.9 Hz, *J* = 5.3 Hz), 1.92 (d, 1H, *J* = 10.52 Hz), −2.79 (s, 2H, NH); ^{13}CNMR (75 MHz, DMSO) δ = 181.94 (C=O), 155.43, 153.56, 149.37, 148.82, 147.05, 139.05, 136.92, 135.8, 132.05, 126.63, 42.83, (C), 133.83, 133.24, 130.82, 129.69, 128.37, 128.23, 125.32, 125.17, 118.23, 117.8, 117.46, 115.03, 112.01, 108.75, 91.54, 67.43, 58.78, 52.46, 40.82 (CH), 45.62, 35.45, 22.65 (CH_2), 42.52 (N—CH_3); (ESI): *m*/*z*(%) 925 (100) [M^+]; Anal. Calcd. for $C_{61}H_{47}N_7O_3$ (926.07): C 79.11, H 5.12, N 10.59; Found: C 79.03, H 5.01, N 10.49.

5-(6-Acetylmorphine-2-yl-azophenyl)10,15,20-triphenylporphyrin (11). Yield: 983 mg (85%), mp = 219–221°C, R_f = 0.42 as a voilet solid; IR (KBr) ν: 3384, 3377 (OH), 3310 (CH), 2989, 2927 (NH), 1722 (C=O acetyl), 1638 (C=C, alkene), 1625 (C=O hydrazone), 1604(C=N), 1525 (C=C, aromatic), 1280, 1087 (C—O—C) cm^{-1}; ^{1}HNMR (300 MHz, DMSO): δ = 13.35 (br s, 2H, NHO), 8.95–8.60 (m, 8H, β-pyrrole), 8.25–6.94 (m, 19H, H_{arom}), 6.83 (s, 1H, aromatic H), 5.56 (d, 1H, *J* = 8.5 Hz, CH=CH), 5.37 (d, 1H, *J* = 8.5 Hz, CH=CH), 4.52 (d, 1H, *J* = 7.8 Hz), 4.32–4.27 (m, 1H), 3.38–3.33 (m, 1H), 3.04 (d, 1H, *J* = 15.35 Hz), 2.59–2.64 (m, 1H), 2.52 (d, 1H, *J* = 7.4 Hz), 2.45 (dd, 1H, *J* = 4.85 Hz), 2.32 (s, 3H, NCH_3), 2.25 (dd, 1H, *J* = 4.6 Hz), 2.14 (s, 3H, CH_3), 1.95 (td, 1H, *J* = 9.11 Hz, *J* = 5.25 Hz), 1.87 (d, 1H, *J* = 10.29 Hz), −2.79 (s, 2H, NH); ^{13}CNMR (75 MHz, DMSO) δ = 183.67, 172.54 (C=O), 155.45, 154.06, 149.49, 148.85, 147.25, 139.15, 136.87, 135.82, 132.35, 127.75, 42.65, (C), 133.73, 133.26, 130.89, 129.73, 128.25, 128.21, 125.37, 125.36, 118.22, 117.85, 117.29, 115.11, 112.14, 108.76, 91.55, 67.45, 58.77, 52.49, 40.85 (CH), 45.64, 35.46, 22.68 (CH_2), 42.22, 23.19 (N—CH_3, $COCH_3$); (ESI): *m*/*z*(%) 967 (93) [M^+]; Anal. Calcd. for $C_{63}H_{49}N_7O_4$ (968.11): C 78.16, H 5.10, N 10.13; Found: C 78.02, H 5.07, N 10.09.

Biological studies. A 500 mg Bond Elut SPE column was used for the extraction. The SPE columns were conditioned by

Scheme 1



the sequential passage of 2 × 3 mL of methanol, 2 × 3 mL of water, and 2 × 5 mL of water adjusted to pH 9.5 with NH_4OH. Ten millilitres of human urine sample adjusted to pH 9.5 with NH_4OH was vortex, centrifuged, and applied to the SPE columns at a rate of 1.0 mL/min. The columns were washed with 2 × 5 mL of distilled water and left to dry for 10 min. The drugs were eluted with a solution consisting of a single phase mixture of dichloromethane/acetone (50/50) and collected in glass tubes. The elution solvent was evaporated to dryness under a nitrogen stream. The dried residues were then reconstituted in slightly warm water, and derivatization was carried out and then the samples were analyzed using AP/ACE MDQ CE system coupled with photo-diode array detectors (PAD).

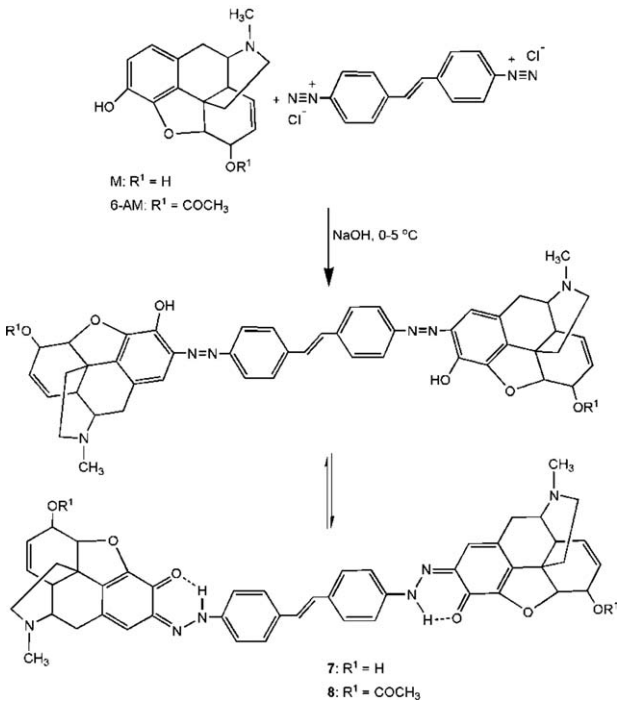
RESULTS AND DISCUSSION

Synthesis. Our straightforward synthesis of morphine azo dyes (**1**–**6**) is outlined in Scheme 1. In a first step, the diazonium ions of aniline derivatives were generated with sodium nitrite in 1*N* HCl. The diazonium ions were then coupled by nucleophilic substitution with the corresponding substrates M or 6-AM. Azo coupling reactions were performed using the diazonium salts of 4-aminosulfonic acid, 3-aminobenzoic acid, 4-methoxyaniline, and 4-nitroaniline to yield 2-(arylazo)morphines **1**–**6**, respectively, (Scheme 1). No reaction was found, however, to occur with codeine under the same reaction conditions.

Synthesis of fluorescent azostilbene morphine dyes was achieved by reaction of *trans*-4,4′-diazostilbene dihydrochloride with M or 6-AM in 1:2 stoichiometric ratio to give stilbene based azo dyes containing two M (**7**) or two 6-AM (**8**) moieties as shown in Scheme 2. The resulting bis-azo dye are belong to the class of direct dyes [31,32]. Furthermore, we have established that *5*-(*p*-aminophenyl)-10,15,20-triphenylporphyrin (**9**) [30] is readily diazotized with sodium nitrite in aqueous mineral acid solution. The diazonium salt obtained is fairly stable; it decomposes significantly at temperature greater than 25 °C. The reaction of porphyrin diazonium salt with M or 6-AM leads to porphyrins containing residues of azo dyes in *meso* position of **10** or **11**, respectively (Scheme 3). The resulting colored compounds were purified by flash column chromatography using hexane/ethyl acetate (2:1) as eluent to produce azo-M (**1**–**4**, **7**, and **10**) and azo-6-AM (**5**, **6**, **8**, and **11**) with excellent yields. Azo coupling reactions of morphines occur predominately ortho to the electron donating hydroxyl group of the morphine aromatic ring. Hence, the inclusion of this design motif in the target dyes avoids potentially difficult separation of isomers.

Spectroscopic studies. Overall characterization of dye structures was carried out by elemental analysis, NMR, UV–vis, IR, and mass spectrometry (see experimental section for details). The NMR spectra of compounds **1**–**8**, **10**, and **11** are consistent with proposed structures, showing the expected features with correct integration ratios. Both ^1H and ^{13}C NMR spectra indicated the appearance of new signals corresponding to the aryl moiety of each azo-compound (Fig. 2). Spectral

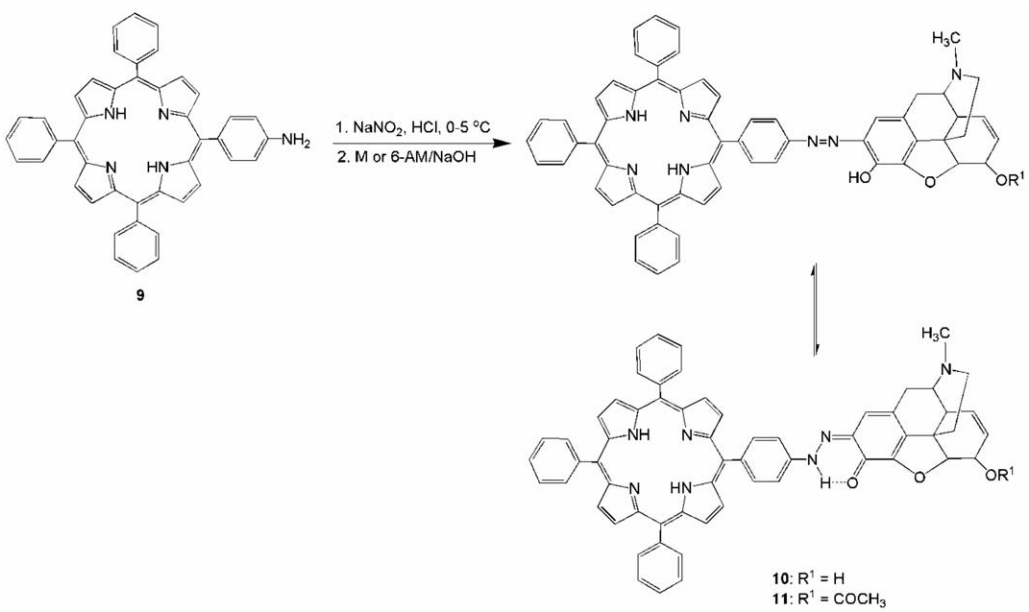
Scheme 2



properties of synthesized dyes were affected by intramolecular hydrogen bond between the phenolic hydroxyl group of morphine moiety and the central nitrogen atom of the azo bridge of the azo dye residues. Azo dyes in which the azo group is conjugated with a hydroxyl group can exhibit azo-hydrazone tautomerism, and NMR spectroscopy is established as an effective technique to study tautomer composition [33–35]. The intramolecular hydrogen bond ring is essentially planar and coplanar with its adjacent phenyl ring, which stabilized the hydrazone form. 2-Arylazomorphine derivatives (dyes **1**–**8**, **10**, and **11**) exist predominantly in the hydrazone form via intramolecular hydrogen bonds, which result in the linearity and coplanar conformation of the dyes [28]. The proton peaks involved in hydrogen bonds appear at much lower field than normal proton peak of hydroxyl group and these (12.75–13.52 ppm for dyes **1**–**8**, **10**, and **11**) were confirmed by ^{1}H NMR. The downfield position of the resonance from the hydrazone proton is attributed to internal hydrogen bonding in which the carbonyl oxygen is hydrogen bonded to this proton [33]. Dyes that occur as the azo tautomer show a ^{13}C resonance at *ca*. 160 ppm from the carbon attached to the phenolic hydroxyl group, whereas those that occur as the hydrazone tautomer show a resonance at *ca*. 180 ppm for the same carbon atom within a carbonyl group (Fig. 2). Dyes that occur as both tautomers show a single resonance between these limits, due to rapid tautomerisation, with the position determined by the relative concentrations of the two tautomers [34–36]. ^{13}C NMR spectra from DMSO samples of **1**–**8**, **10**, and **11** confirmed the hydrazone structure by detecting a new carbonyl peak at 181–183 ppm assigned to C3 of M or 6-AM and, thus, morphine dyes **1**–**8**, **10** and **11** are present as the hydrazone tautomer (*ca*. 100%).

According to DFT calculations at the B3LYP/6-31G* level, the hydrazono toutomers of **1**–**8**, **10**, and **11** are

Scheme 3



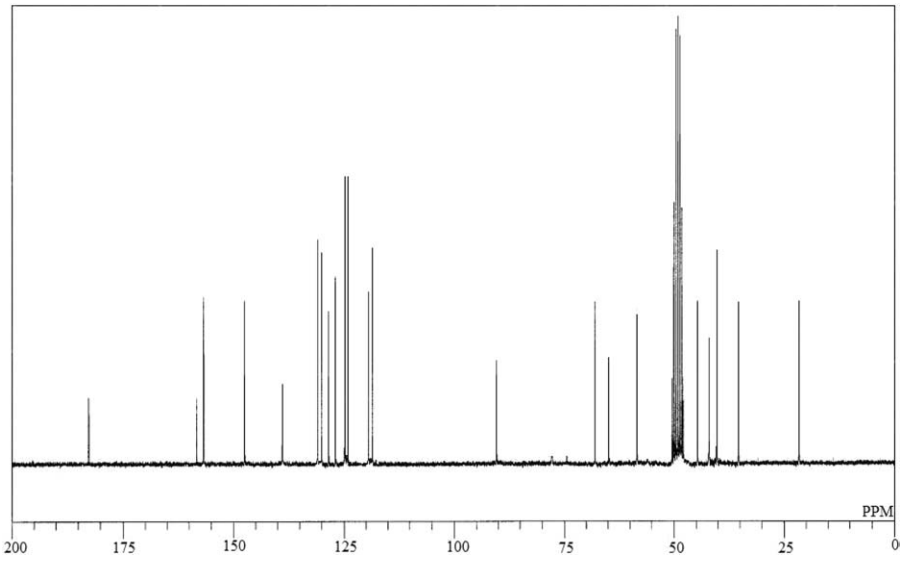


Figure 2. ^{13}C NMR spectrum of compound **3** in DMSO(d_6) at 20°C.

favored over the azo toutomers by 2.4–3.6 kcal mole^{-1}. As major entropy differences and crystal lattice effects are not to be expected here, these results should secure the hydrazone formulas. Consequently, it can be considered that morphine azo dyes (**1–8**, **10**, and **11**) have such structures as shown in Schemes 1–3.

IR spectra assigned with the aid of the NMR data, provide fingerprints for hydrazone form and hydrogen bonding. The IR spectra of all the resulting colored compounds confirmed the presence of a C═O bond which resonates at 1625–1618 cm^{-1}. IR spectrum of compound **4** showed absorption bands at 1517 and 1334 cm^{-1} due to the presence of a NO_2 group, whereas the SO_2 group of compound **1** and **5** has two vibrational frequencies at 1350 and 1150 cm^{-1}. Moreover, the vibrational frequency of aliphatic OH band ν(O─H) of M or the $COCH_3$ band ν(C═O) of 6-AM was not found to be sensitive to the connection of M or 6-AM with the azo derivatives. For compounds **1–4** and **7** ν(O─H) lies in the range of 3385–3375 cm^{-1}, and for compounds **5**, **6** and **8** ν(C═O) from 1730 to 1718 cm^{-1}, comparable to the frequencies of M ν(O─H) at 3373 cm^{-1}, and 6-AM ν(C═O) at 1713 cm^{-1}, indicating that the intra bonding of the morphine moiety was not perturbed by substitution on the phenyl ring.

Evidence in support of structures **1–8**, **10**, and **11** is presented by mass spectrometry. The ESI-MS spectra of these compounds in MeOH showed molecular ion peaks (M^+) corresponding to the formula of each compound. Mass spectra of all compounds showed molecular ion peaks corresponding to their expected pattern of abundance ranging from 85 to 100% (Fig. 3).

The electronic absorption spectra (EAS) of the investigated morphine dyes **1–8**, **10**, and **11** in ethanolic solutions were studied. There is no visual evidence for a band around 380–420 nm, which could be assigned to the azo form. The compounds comprised two to three bands in the UV region and one band in the visible region (Fig. 4). The band of shortest wavelength appearing in the range 210–255 nm was best ascribed to π–π* transition of the benzenoid system of the compounds. The second band observed in UV region, in the wavelength range 270–285 nm was attributed to π–π* transition within the furan heterocyclic moiety of the compounds. The third band observed in the UV region at 285–290 nm was assigned to n–π* electronic transition

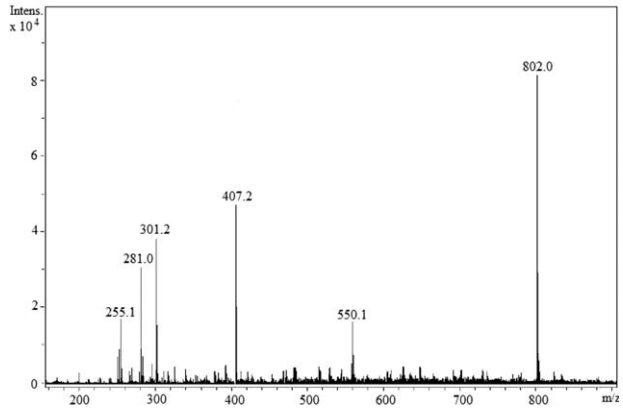


Figure 3. ESI-MS of compound **7** in CH_3OH.

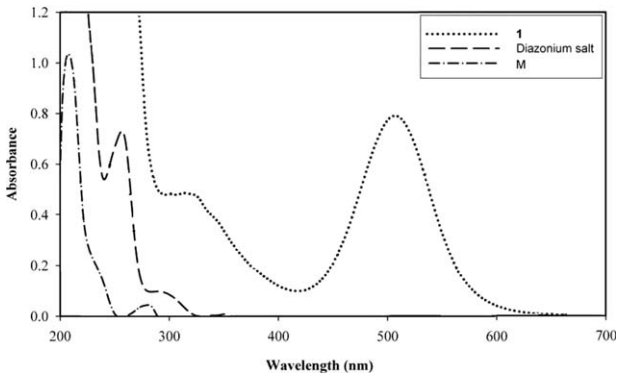


Figure 4. Electronic absorption spectra of 2×10^{-5} mol dm^{-3} of **1**, diazonium salt of sulfanilic acid and M.

of OH groups. The long wavelength band at about 510 nm corresponds to the hydrazone form [37] (Fig. 4). This band was capable of being assigned to π–π* transition involving the whole electronic system of the compounds with a considerable charge-transfer (CT) character. Such a CT originated mainly from the aryl azo to the morphine moiety, *i.e.*, this band was due to intramolecular CT transition. When analyzing EAS of the porphyrin azo dyes (**10** and **11**), it was difficult to draw an unambiguous conclusion as to whether the π system of the azo dye interacts with the π system of porphyrin ring. The Soret band of tetraphenyporphyrin ($\lambda_{max} \sim$ 400 nm; $\varepsilon \sim 4.75 \times 10^5$ dm^3 mol^{-1} cm^{-1}) is found alongside with the broad absorption band of azo dye residue ($\lambda_{max} \sim$ 590 nm; $\varepsilon \sim 3.35 \times 10^4$ dm^3 mol^{-1} cm^{-1}), which does not permit a confident judgment to be made on whether transfer of π electron density from the azo dye residue to the porphyrin ring has taken place. However, the sharp reduction in intensity of the Soret band and the growth in intensity of the electronic transition bands and also their bathochromic shift indicate the existence of such interactions.

The compounds (**1**–**8**) exhibited emission fluorescence peak even at very low concentration (5–8 $\times 10^{-9}$ mol dm^{-3}) in aqueous solution as indicated by capillary electrophoresis (CE) with UV-fluorescence photo diode-array detectors. However, compounds **10** and **11** showed highly intense fluorescence peaks at 665 and 670 nm, respectively. The synthesis of **10** and **11** will provide new insight into the role of morphine determination using simple and fast chemistry as well as highly sensitive techniques [*e.g.*, CE with laser induced fluorescence detector (LIF)].

Biology. The determination of M in biological samples has become almost a routine assay in many toxicology laboratories owing to the spread of the abuse of

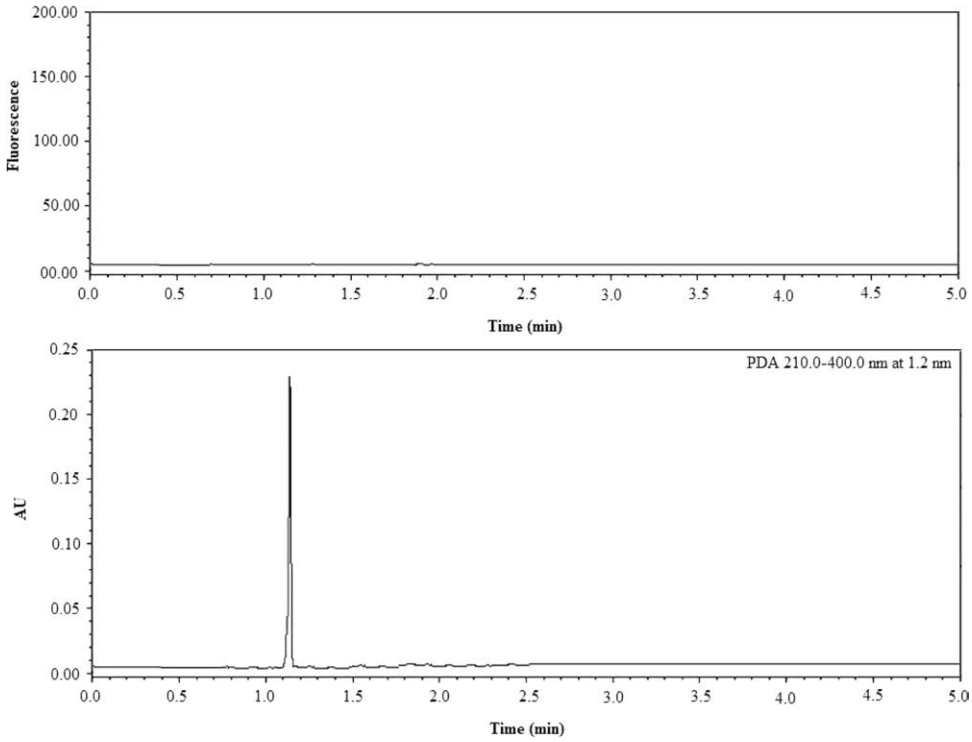


Figure 5. Electropherograms of 5×10^{-6} mol dm^{-3} diazonium salt of sulfanilic acid in water under the optimized conditions: 10.0 s injection time, applied voltage 25 kV, 25°C, 100 mmol dm^{-3} borate electrolyte concentration, and pH 9.0.

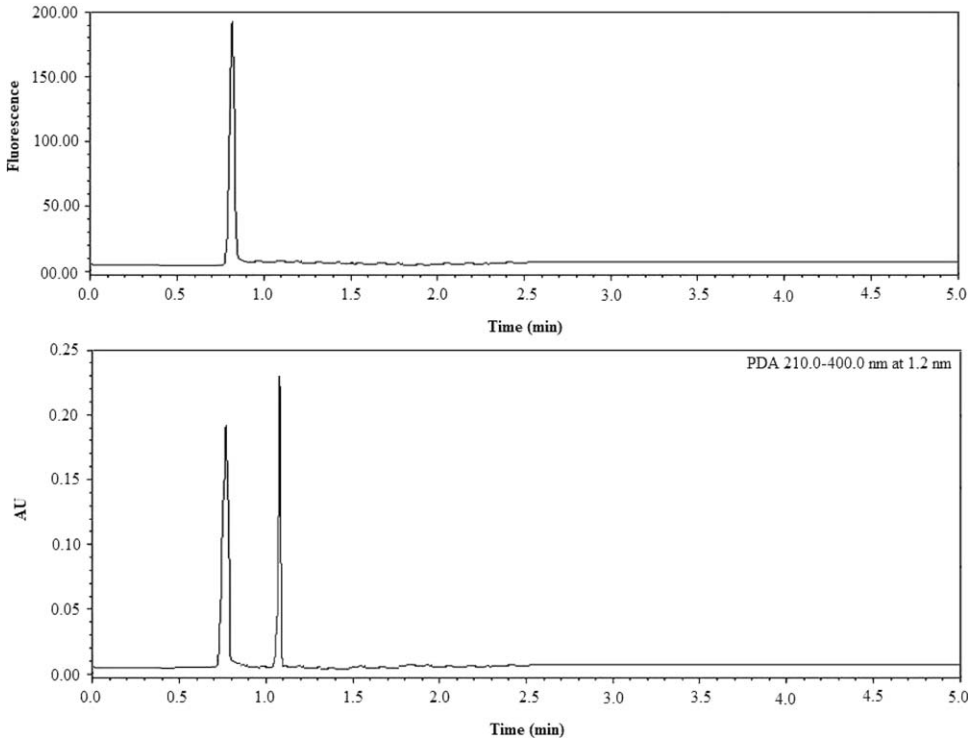


Figure 6. Electropherograms of human urine sample spiked with 5×10^{-9} mol dm^{-3} of M coupled with 5×10^{-6} mol dm^{-3} diazonium salt of sulfanilic acid under the optimized conditions: 10.0 s injection time, applied voltage 25 kV, 25°C, 100 mmol dm^{-3} borate electrolyte concentration, and pH 9.0.

heroin, which is mainly biotransformed into M. In this experiment, the coupling reaction of diazonium salt of sulfanilic acid was carried out with drug-free urine sample and with urine sample spiked with M and 6-AM. No remarkable change was observed for the drug-free urine sample as indicated by CE (data not shown). For urine sample spiked with the M, a deep red color appeared at once which was measured by CE, giving two peaks after 45 s and 65 s corresponding to azo-M (**1**) and diazonium salt of sulfanilic acid, respectively, (Figs. 4 and 5). The extraction recoveries were found to be >99.5% and RSD values of the recovery did not exceed 0.92% indicating good repeatability of the adopted method.

CONCLUSIONS

A number of M and 6-AM azo dyes were synthesized and the possibility of using these dyes as color chemosensors of abused drugs is reported. The synthesis starts from commercially available aniline derivatives and can be completed in one step with an overall yield of 76–92%. *Trans*-4,4′-diaminostilbene or 5-(*p*-aminophenyl)-10,15,20-triphenylporphyrin in azo dye reaction gave highly fluorescent morphine dyes which could be easily detected at very low concentrations using CE techniques. It is found that between the phenolic OH and the central N atom intramolecular proton transfer exists with the hydrazone form being major component. The compounds existed in hydrazone forms exclusively, being stabilized by the intramolecular hydrogen bonds. The resulting azo compounds are highly fluorescent in ethanol and water. Low detection limit was obtained ranging from 5–8 nmol dm^{-3} for M or 6-AM coupled with freshly prepared diazonium salts. Consequently, this method is characterized by simple, rapid, and economic determination of abused drugs in forensic cases as an initial test and clinical analysis to prevent overdose-induced toxicity.

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