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Separation and isolation of tautomers of 2-hydroxy-4-naphthoquinone-1-oxime derivatives by liquid chromatography: Antiproliferative activity and DFT studies

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Abstract. Reversed phase HPLC separation and isolation of isomers of 2-hydroxy-4-naphthoquinone-1-oxime (Lwox) and 3-methyl-2-hydroxy-4-naphthoquinone-1-oxime (Phox) have been investigated. Two distinct peaks are observed in the chromatogram of Lwox and are assigned to 'para' tautomer; 2-hydroxy-4-naphthoquinone-1-oxime (**3**) and 'ortho' tautomer; 4-hydroxy-2-naphthoquinone-1-oxime (**4**). The tautomeric equilibrium of **3** and **4** has been manipulated by incrementally increasing the pH of the mobile phase from 2.5 to 10.5, and altering the solvent polarity. At pH > 6.8 the tautomers are well-separated from each other. There is no separation of Phox isomers between pH 2.5 and 10.5. Isolation of the tautomers has been carried out by preparative HPLC, with **3** and **4** obtained as ammonium bicarbonate adducts and characterized by LC-MS, FT-IR, and UV-visible spectroscopy. Red-orange **3** is characterized by a paranaphthoquinone stretch at 1287 cm^{-1} and a charge transfer band at 420 nm; yellow **4** exhibits, a similar stretch at 1246 cm^{-1} and absorption band at 406 nm. Compounds **3** and **4** were screened for selective antiproliferative activity in three cancer cell lines of different tissue types (COLO 205 (human colorectal adenocarcinoma), U87 MG (glioblastoma astrocytoma) and MIAPaCa-2 (human pancreatic carcinoma). Geometry-optimized structures for tautomers **3** and **4** (**3′** and **4′** in Phox) were computed using the B3LYP method. Structures, **3** and **3′** are 4.7 and 5.8 kcal mol^{-1} more stabilized than **4** and **4′**, respectively, as a result of a hydrogen bond interaction between the 2-hydroxyl group and the nitrogen of the oxime.

Keywords. Naphthoquinoneoximes; tautomer; reversed phase separation; DFT; naphthoquinone; HPLC.

1. Introduction

1,2-Benzoquinone monooximes, 1,2-naphthoquinone monooxime (nitrosonaphthol) and 1,2-anthroquinone oxime show tautomeric equillibria in solution between nitroso and oxime form. In 1,2-benzoquinoneoximes, the nitroso tautomeric form is more stable,[1] while in 1,2-naphthoquinoneoximes, oxime form is more stable.[2] The tautomeric forms of 1 and 2-nitrosonaphthols have been extensively studied by spectroscopic techniques viz UV-visible, IR and NMR spectroscopy.[3–6] In nitrosonaphthols, the presence of two distinct species (A and B, scheme 1) are related through an intramolecular proton transfer through the existing intra molecular hydrogen bond. Oxime tautomers of A and B are more stable than nitroso form.[2] According to X-ray crystal data,[7] *syn* oxime tautomer is present in solid state. However, in solution both the oxime and nitroso tautomeric forms coexist. Experimental and theoretical results confirmed the presence of *syn* and *anti* oxime isomers in nitrosonaphthol.[2] Theoretical studies on this equilibrium between the nitrosonaphthol and oxime form are concentrated on total energies of tautomers with intramolecular hydrogen bonding.[8] Calculated total energies indicate that quinonoid form to be more stable by 2.68 and 2.64 kcal mol^{-1} by DFT and MP2 methods, respectively.[8] The barriers of tautomerization in gas phase for A and B are estimated to be 4.70 and 5.48 kcal mol^{-1}, respectively.[2]

Stereoisomer's of hydroxy-naphthoquinoneoxime and its C3 derivatives (scheme 2), along with their

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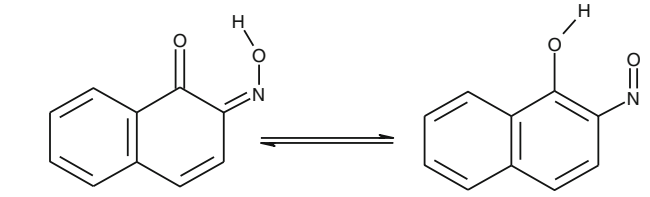
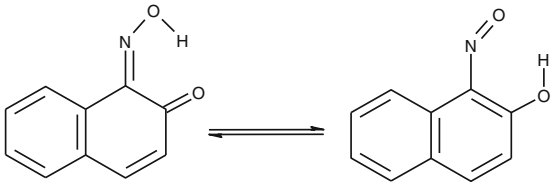
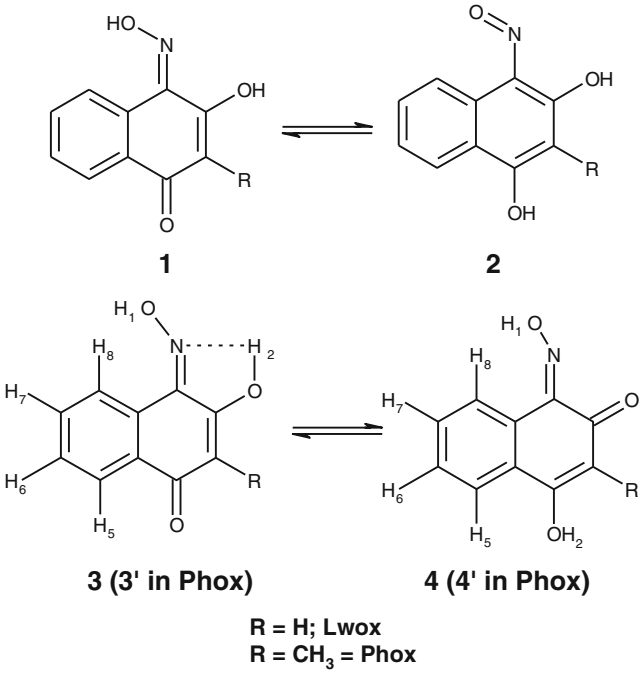


Tautomers of 2-nitroso-1-naphthol (A)



Tautomers of 1-nitroso-2-naphthol (B)

Scheme 1. Tautomers of 1- and 2- nitrosonaphthols.



Scheme 2. Tautomers of hydroxy-naphthoquinoneoximes.

nitrosophenol tautomers, were defined by ^{1}H NMR spectroscopy[9] and it has been confirmed that the relative abundances of *syn* and *amphi* stereoisomer's is governed by steric as well as hydrogen bonding interactions. Single crystal X-ray diffraction studies of 3-methyl-1,4-naphthoquinone-1-oxime[10] and 3-halo-1,4-naphthoquinone-1-oxime[11] show *amphi* isomers as the dominant form in solid state. Vibrational frequencies of 2-hydroxy-1,4-naphthoqunone-1-oxime and its C3 derivatives (CH_3, NH_2, Cl, NO_2) in keto and nitrosonaphthol are known.[12] Among all derivatives (except nitro) the *amphi* conformer in the keto form is predicted to be most stable, which is attributed to the intramolecular hydrogen bonding. It is well-known that hydroxy naphthoquinones possess *ortho* and *para* tautomeric forms in solution.[13,14]

Keto-enol tautomerism in oximes has been studied by gas chromatography,[15,16] however separation and isolation of these tautomeric naphthoquinoneoximes still not established. Here, we detail the separation, isolation and characterization of tautomers of 2-hydroxy-4-naphthoquinone-1-oxime (Lwox) and 3-methyl-2-hydroxy-4-naphthoquinone-1-oxime (Phox) shown in scheme 2. The separated tautomers **3** and **4** of Lwox were screened for anti-proliferative activity in three cancer cell lines of different tissue types (COLO 205, U87 MG and MIAPaCa-2). The optimized structures of tautomers **3**, **3′**, **4** and **4′** have been evaluated by DFT employing a dielectric continuum to model solvent effects. The calculations identify a hydrogen bond between the 2-hydroxyl (in **3** and **3′**) and the oxime nitrogen that stabilizes this conformation over the 4-hydroxyl (i.e., **4** and **4′**) tautomer.

2. Experimental

2.1 *Chemicals and materials*

HPLC grade CH_3CN and CH_3OH was obtained from Merck chemicals. HCl, TFA, hydroxylaminehydrochloride, NaOH, ammonia, ammonium acetate, ammonium bicarbonate, ammonium formate and formic acid have been obtained from Qualigen Chemicals, India. 3-Methyl-1,4-naphthoquinone (menadione), 2-hydroxy-1,4-naphthoquinone (lawsone) has been obtained from Sigma-Aldrich. Milli Q water is used wherever necessary, anhydrous methanol is prepared for synthesis of ligands.[17]

The starting materials, 2-hydroxy-3-methyl-1,4-naphthoquinone (phthiocol) and 2-hydroxy-4-naph thoquinone-1-oxime (Lwox), 3-methyl-2-hydroxy-4-naphthoquinone-1-oxime (Phox) have been prepared according to published procedures.[10,18]

2.2 *Apparatus*

Shimadzu, LC-2010C_{HT} liquid chromatograph has been used for separation of tautomers. The detector used is SPD-M20A diode array and auto sampler injection system, connected with LC-solution with a multichannel module. Shimadzu LC-8A preparative liquid chromatograph has been used for isolation of tautomers. The detector used is SPD-M20A diode array detector and a rheodyne valve manual injection system

(5000 μL loop), connected with LC-Solution with a multi-channel module.

Liquid chromatograph mass spectrums were recorded on Shimadzu, LCMS-2010EVwith ESI source has been used for ionization. FT-IR spectra's was recorded on a SHIMADZU FT 8400 spectrometer in KBr pellets.

2.3 *Chromatography*

An YMC ODS-A C18 and X-bridge C18 column [length = 150 mm, internal diameter = 4.6 mm, particle size = 5 μm, pore size = 12 nm] has been used for separation of tautomers. A YMC-ODS C18 column [length = 500 mm × 30 mm, particle size = 10 μm] has been used for isolation of tautomers. The mobile phase consisted of water: acetonitrile in various proportions. Water and acetonitrile is always acidified with trifluoroacetic acid (TFA), mixture of ammonium formate and formic acid, basified by using ammonium bicarbonate, mixture of ammonium formate and ammonia. Ammonium acetate is used for preparation of neutral mobile phase. Detector was set at 254 nm.

2.4 *Separation and isolation of tautomers*

Following experimental conditions are used for separation and isolation of naphthoquinoneoxime derivatives. Method I to method VII has been used for separation of isomers/tautomers. Method VIII is used for isolation of isomers of Lwox respectively. Method IX has been used for LC-MS analysis of Lwox (see Supplementary information).

2.5 *Sample preparation*

500 ppm and 5000 ppm solution of Lwox and Phox has been prepared in methanol for separation and isolation, respectively of tautomers.

2.6 *Cell lines*

COLO205 (human colorectal adenocarcinoma), U87MG (human primary glioblastoma) and MiaPaCa-2 (human pancreatic carcinoma) cell lines were obtained from National Centre for Cell Science (Pune, India). COLO205 cell line was cultured routinely in RPMI-1640 medium (HiMedia, India) while U87MG and MIAPaCa-2 in DMEM media. Both medias were supplemented with 2 mM glutamine (HiMedia, India), antibiotics (100 U/mL penicillin A and 100 U/mL streptomycin; HiMedia, India) and 10% heat-inactivated fetal bovine serum (HiMedia, India). All cell lines were cultured in 25 cm^2 flasks with loosened caps and incubated in humidified air containing 5% CO_2 at 37°C.

2.7 *Antiproliferative activity studies*

The effect of isolated tautomer **3** and **4** on the viability of the cell lines were measured using XTT assay. The antiproliferative activity was evaluated based on the amount of 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt formed by the viable cells in the treated wells. Fresh stock solutions of **3** and **4** were prepared in DMSO at a concentration of 100 mM. Serial dilution in 50:50 media: DMSO mixtures produced stock solutions of compounds ranging from 10^{-8} M to 10^{-4} M. About 50 μL of the cell suspension diluted to a final density of 1×10^5 cells/mL were sowed into each well of a 96-well culture plate (Axygen, USA) and treated with the varying concentrations of compounds in duplicates. The 96-well plates were left for incubation with compounds at 37°C, 5% CO_2 in humidified atmosphere for 72 h. XTT reagent was freshly prepared with XTT-labelling reagent and electron-coupling reagent in a ratio of 50:1. After 72 h of incubation, 50 μL of this mixture was added to each of the 96 wells. The plates were incubated at 37°C, 5% CO_2 in humidified atmosphere and read out after optimal colour development in each of the wells. Quantification of cell viability was performed in an ELISA plate reader (Bio-Rad, München, Germany) at 490 nm with a reference wavelength of 655 nm. The above-mentioned cell lines treated with doxorubicin served as a positive control for cytotoxicity and to evaluate the same.[19] OriginPro software and MS-Excel were used for data analysis and determination of IC_{50} value.

2.8 *DFT calculations*

All DFT calculations were performed with the ORCA program.[20] Geometry optimized structures of **3** and **4** (**3′** and **4′** in Phox) were achieved using the B3LYP method.[21,22] The conductor-like screening model (COSMO) was applied using water as the solvent.[23] The all-electron basis sets of triple-ζ quality were those developed by the Ahlrichs group.[24,25] Auxiliary basis sets were used to expand the electron density in the calculations were chosen to match the orbital basis. Geometry search was carried out in redundant internal coordinates without imposing geometrical constraints, and stationary points were characterized via analytical frequency calculations.

3. Result and discussion

3.1 *Separation of 2-hydroxy-4-naphthoquinone-1-oxime (Lwox) isomers*

Separation of Lwox isomers was achieved varying the pH of the mobile phase using appropriate buffer solutions. Chromatographic separation is based on gradient elution. Figure 1 shows a chromatogram of Lwox separation obtained at pH 7 (without modifier), 2.5, 4.5, 6.8, 8.5 and 10.5 of mobile phase with chromatographic data is summarized in table S1. (i) At pH 7, it has been observed that there is no interaction of Lwox (figure 1a) with mobile phase as well as stationary phase, Lwox retains in the column, moreover this pH is not suitable for the separation and elution of isomers/tautomers. Either Lwox is unstable in solution or there may be fast inter conversion of isomers at pH 7. (ii) At pH 2.5 and 4.5 (figure 1b and c) peak shapes is similar to equilibrium of the isomers.[26] There is no separation observed between the isomers/tautomers at this pH. (iii) At pH 6.8 of the mobile phase, two peaks were observed that were well-resolved from each other (figure 1d). We named these two peaks as isomer X and

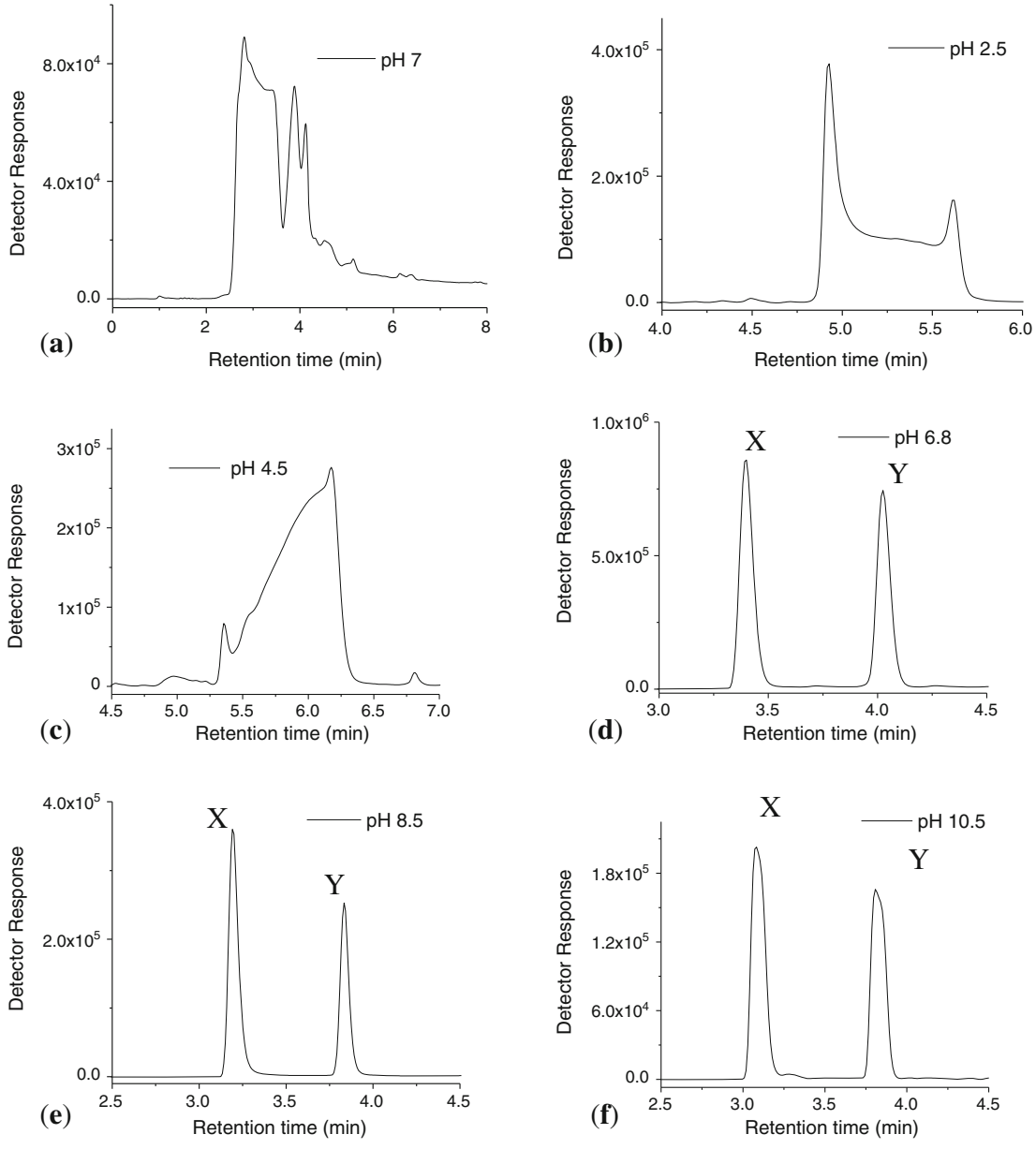


Figure 1. HPLC chromatogram of Lwox ligand; (**a**) in neutral pH, (**b**) pH 2.5, (**c**) pH 4.5, (**d**) pH 6.8, (**e**) pH 8.5 and (**f**) pH 10.5.

Table 1. Chromatographic data for peaks observed in figure 1e and f at 254 nm.

pH	Peak	Ret. time (in min.)	% Area	Peak purity index
8.5	X	3.426	57.198	0.997
	Y	4.073	42.802	0.998
10.5	X	3.079	53.792	1.000
	Y	3.805	45.104	0.999
Isocratic	X	2.612	53.865	1.000
	Y	4.200	46.135	1.000

isomer Y (the peak with less retention time is named as isomer X and the other with more retention time is named Y isomer). The separation between the isomer X (Rt-3.392 min) and isomer Y (Rt-4.020 min) is observed to be ∼0.628 min and isomer X is present in more quantity than Y (table S1). The peak purity index has been found to be 0.99 for both the isomer; hence there is no merged impurity in both isomers. (iv) At pH 8.5 pattern of chromatogram (figure 1e) is similar to that of pattern observed at pH 6.8 but the separation of isomers at this pH of mobile phase is much higher (∼0.647 min.) as compared to pH 6.8 (table 1). Peak purity index of both the isomer is 0.99; hence there is no merged impurity. Tailing factor of isomer X and isomer Y is 1.426 and 1.201, respectively, thus the peak shape of isomer X is less symmetric than the isomer Y. (v) At pH 10.5 of mobile phase, two peaks of isomers/tautomers (figure 1f, table 1)

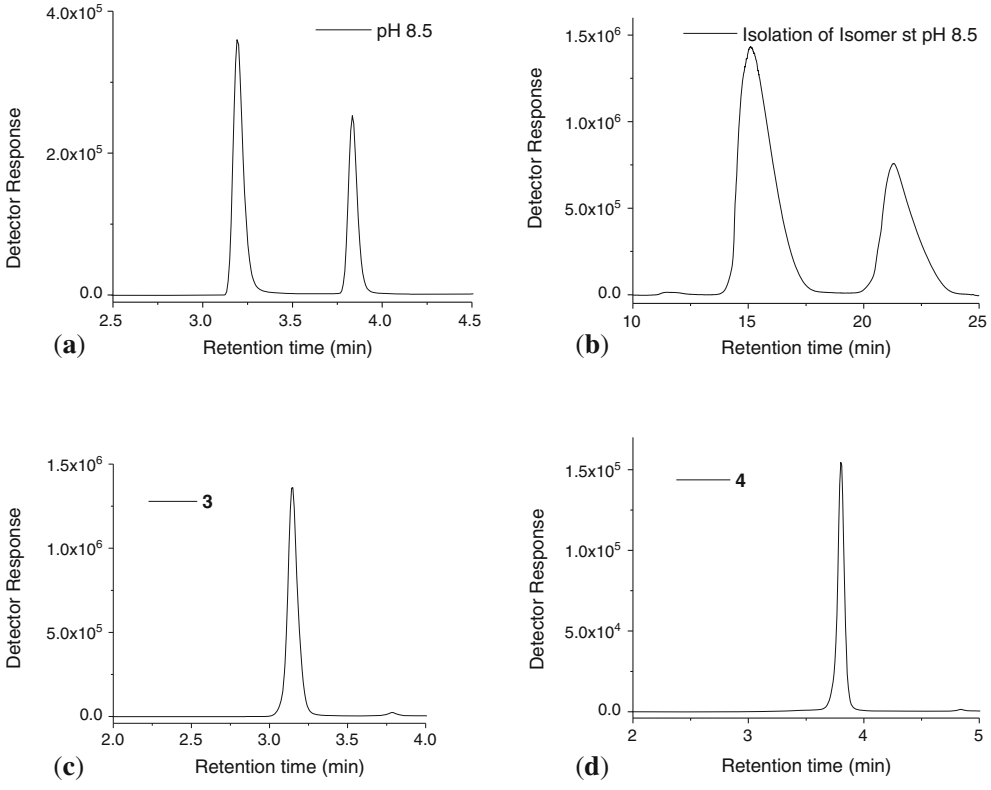


Figure 2. (**a**) Chromatogram obtained before isolation of isomers at pH 8.5. (**b**) Chromatogram obtained for isolation of isomers at pH 8.5 on preparative HPLC. (**c**) Chromatogram of isomer **3** after isolation. (**d**) Chromatogram of isomer **4** after isolation.

were observed with a separation between the peak X (Rt-3.079) and peak Y (Rt-3.805) is 0.72 min. This chromatographic condition is suitable for separation of isomers/tautomers. (vi) Two distinct peaks are observed in isocratic elution. The better resolution is observed of tautomers in isocratic elution in comparison to gradient elution (figure S1). Peak shape is sharp in isomer X compared to isomer Y. The peak shape of isomer Y is broad due to intermolecular or intramolecular hydrogen bonding effects of the carbonyl oxygen's or the hydroxyl group.[10,11] Peak shape of X (tailing factor ~1) isomer was found to be more symmetric than isomer Y (tailing factor ~0.88). Abundance of isomer X is more than isomer Y in isocratic elution (table 1).

When *syn*, *anti* and *amphi* isomers,[9] are present in the solution of Lwox, several peaks are expected[27] in the chromatogram. However, only two peaks are observed at pH 6.8, 8.5 and 10.5. The peak shape for all pH

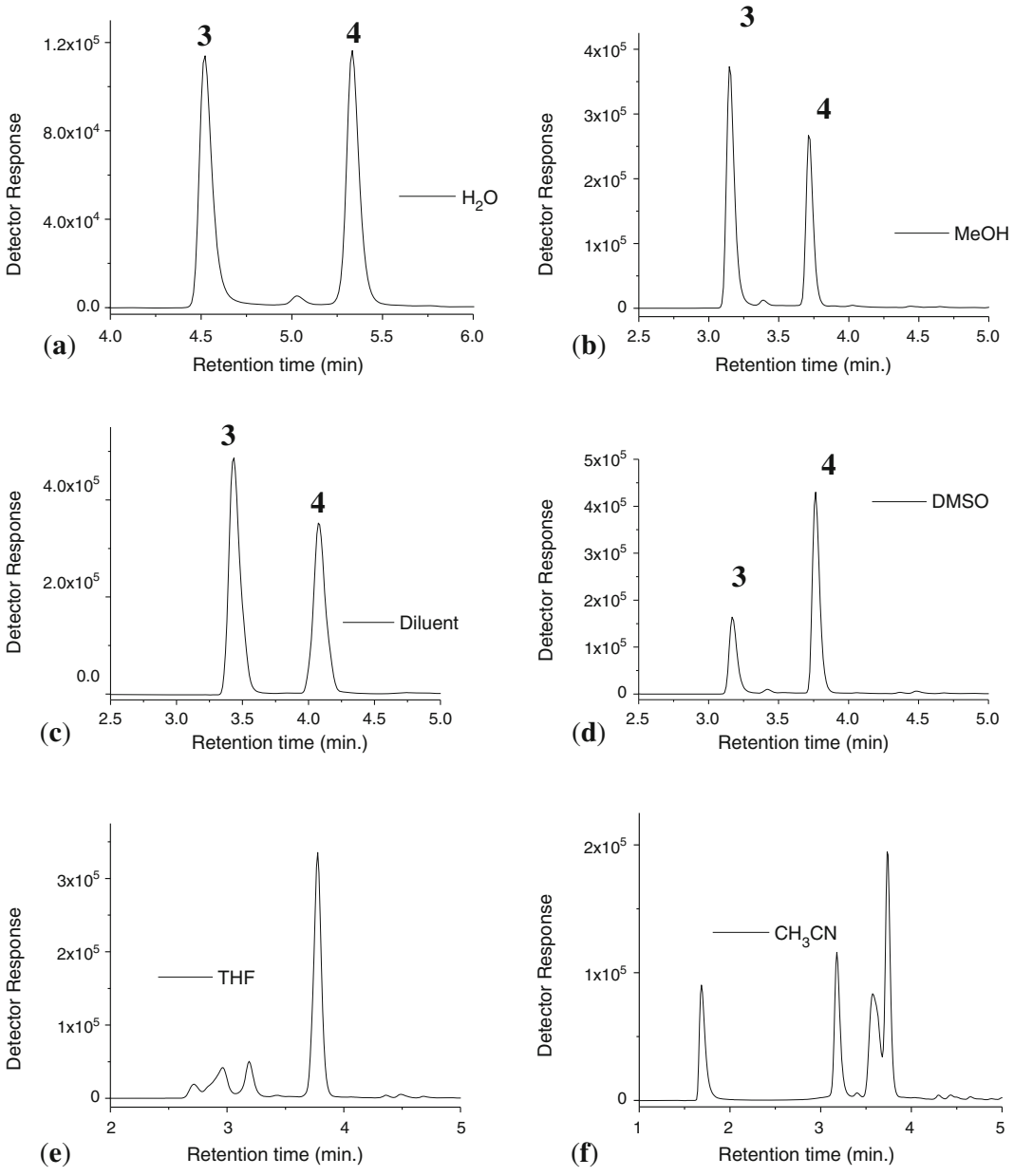


Figure 3. Effect of solvent on retention and separation of isomers of Lwox; (**a**) H_2O, (**b**) CH_3OH, (**c**) CH_3CN, (**d**) THF, (**e**) diluent, and (**f**) DMSO.

range (except pH 7) was observed to be good. The separation in neutral buffer and basic buffer pH range is observed to be base to base, however in acidic pH range there is no base to base separation of the peaks and the nature of the chromatogram observed at pH 2.5 and 4.5 is similar to equillibria found for keto-enol oxime tautomers.[26] Further, the spectroscopic methods like FT-IR and UV-Visible spectra clearly indicated the presence of *ortho* and *para* tautomers[14] of Lwox at pH 6.8, 8.5 and 10.5. Hydroxy naphthoquinones are known to exist in *ortho* and *para* tautomeric forms.[28,29] Peak Y retains longer time on the column, due to the presence of hydrogen bonding effects and polarity, this peak has been assigned to *ortho* i.e., tautomer **4**; (4-hydroxy-2-naphthoquinone-1-oxime), while peak X has been assigned for *para* i.e., tautomer **3** (scheme 2). The tautomer **3**; (2-hydroxy-4-naphthoquinone-1-oxime) is stabilized by strong intramolecular hydrogen bonding hence will not adsorbed strongly on the column and will be eluted before tautomer **4**.

3.2 *Reversed phase chromatographic isolation of tautomers of Lwox*

The chromatogram obtained before isolating the isomers is shown in figure 2a. The % of tautomer **3** have been observed (Rt = 3.140 min) to be more than tautomer **4** (Rt = 3.799 min). The already developed method V has been scale up on preparative HPLC for the isolation of tautomers, the obtained chromatogram of isolation of tautomers is shown in figure 2b. Further details used are mentioned in method VIII. The tautomer **3** (figure 2c) has been eluted at Rt = 15.09 min. and tautomer **4** (figure 2d) eluted at Rt = 21.27 min.

3.3 *Purity of tautomer **3** and **4** after isolation*

A solid of tautomer **3** was obtained after lyophilisation, at low temperature with purity of isolated solid **3** determined to be 98.5% by LC-MS analysis (figure S2). The chromatogram also shows ~1.5% tautomer **4**. The percentage of tautomer **4** increases with time in solvent. The solid of **4** have been obtained after lyophilisation and contains ammonium bicarbonate that is 99.9% pure.

3.4 *LC-MS analysis of Lwox and isolated tautomers **3** and **4***

The tautomer has been confirmed by LC-MS analysis (figure S2). While chromatographic methods I, II, III are not suitable for separation of isomers, methods IV, V and VI are suitable for analysis; however in method IV and V, the peak shape is broad and separation between the peaks is less as compared to method VI. Thus, method IV and V are excluded. The method VI is suitable for LC-MS analysis of tautomers.

The molecular weight of Lwox is 189.17 g mol^{-1} and experimentally it was determined to be 190 [M + 1] by using positive polarity and ESI probe (source for ionization). There is a presence of tautomer **4**, ~13% in isolated solid of **3**. The presence of tautomer **4** is due to interconversion of tautomer **3** during the course of analysis. The purity of tautomer **4** was found to be ~95%.

Table 2. Chromatographic data for peaks observed in figure 3 at 254 nm.

Sl. No.	Solvent	Ret. time (in min.)	% Area	Peak purity index	Tailing factor
1.	H_2O	4.515	50.58	0.999	1.61
		5.327	49.42	0.999	1.30
2.	CH_3OH	3.145	61.35	0.999	1.55
		3.711	38.65	0.999	1.49
3.	Diluent	4.727	47.19	1.000	1.59
		5.552	52.81	1.000	1.31
4.	DMSO	3.165	29.69	0.999	1.56
		3.758	70.31	1.000	1.49
5.	THF	2.710	04.49	1.000	–
		2.957	15.59	0.999	–
		3.184	11.30	0.999	–
		3.770	68.68	1.000	1.08
6.	CH_3CN	1.681	18.49	0.956	2.05
		3.174	21.39	0.999	1.43
		3.571	26.55	0.998	–
		3.733	33.57	0.997	–

3.5 *FT-IR spectra of Lwox and isolated tautomer **3** and **4***

The isolated tautomers **3** and **4** show vibration peaks for ammonium bicarbonate in FT-IR spectrum (figure S3). The vibrational frequency at 1519 cm^{-1} has been assigned to ammonium bicarbonate. The other vibrations of ammonium bicarbonate merged in strong absorption of Lwox tautomers. The nature of the FT-IR spectrum varies between 3600 and 2500 cm^{-1} for **3** and **4** (figure S3a). $\nu_{C=O}$ and $\nu_{C=N}$ vibrations are observed at 1707 $cm^{-1}$and 1585 cm^{-1}, respectively in **3**. A paranaphthoquinone (p-NQ) vibration are observed at 1280 $cm^{-1}$and 1263 cm^{-1} in **3** as expected these frequency were absent in **4**, instead a broad band centred at ∼1246 $cm^{-1}$is observed.

3.6 *UV-visible spectra of isolated tautomer **3** and **4***

The colour of isolated tautomers was red-orange for tautomer **3** and yellow for tautomer **4**. UV-visible

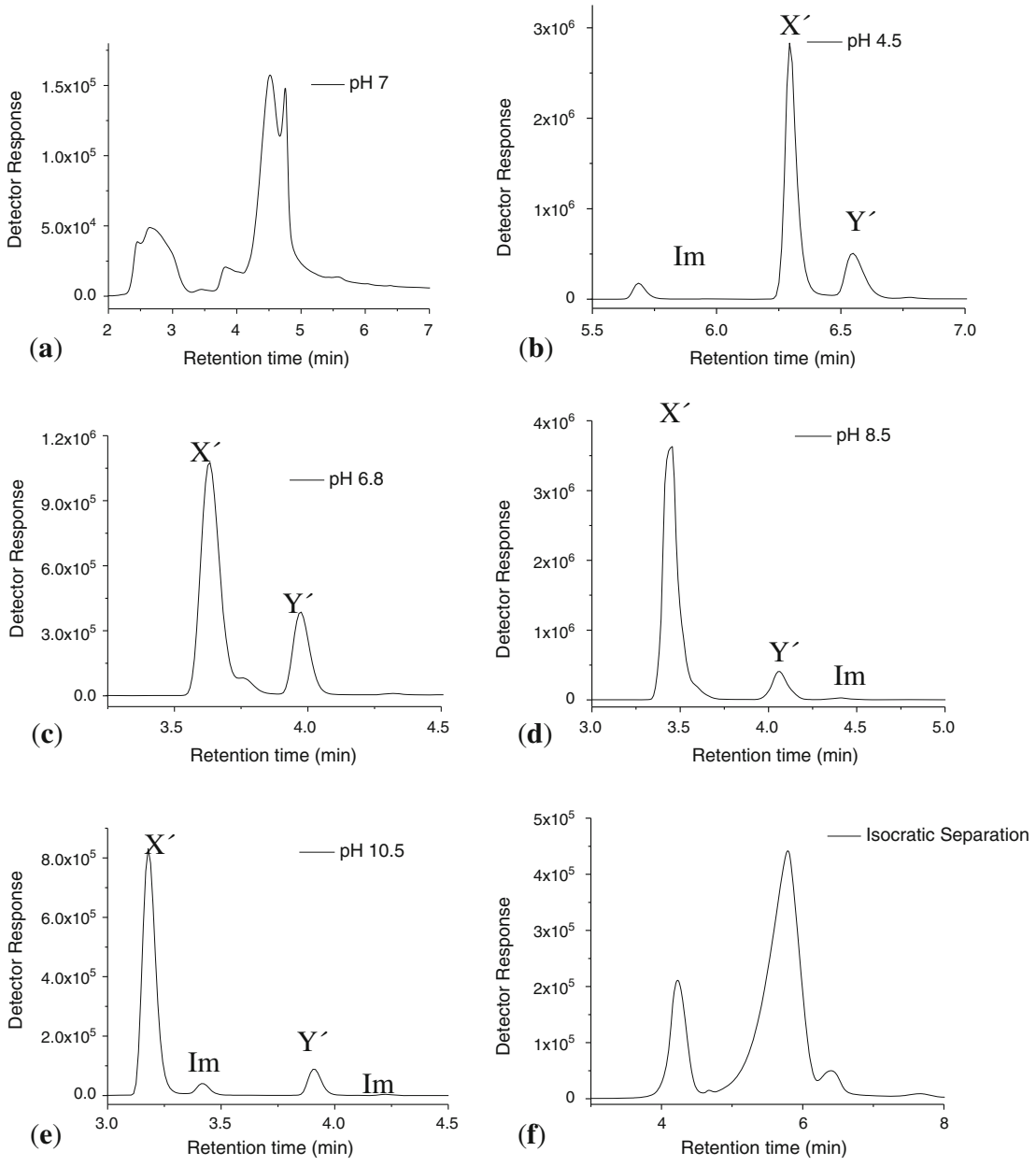


Figure 4. HPLC chromatogram of Phox; (**a**) at neutral pH, (**b**) pH 4.5, (**c**) pH 6.8, (**d**) pH 8.5, (**e**) pH 10.5, and (**f**) isocratic elution.

spectra (figure S4) of **3** shows peaks at 212, 247.5, 262.1, 319.1 nm in UV region and a peak at 420 nm in visible region, while **4** shows peaks at 216.4, 263.8, 290.8, 333.8 and 389.8 nm in UV region and 405 nm in visible region. The peaks in UV region are assigned to $\pi-\pi^*$ transition of the quinonoid and benzenoid rings, and a peak in visible region was classified as a charge transfer transition for in tautomer **3** and **4**.

3.7 *Effect of solvent polarity on separation of the isomers/tautomers of Lwox*

Composition of tautomers varies with polarity of the solvents where as composition of stereoisomer's remains the same. The solution of Lwox has been prepared in various solvents viz H_2O, diluent, DMSO, CH_3OH, THF and CH_3CN. Neutral chromatographic

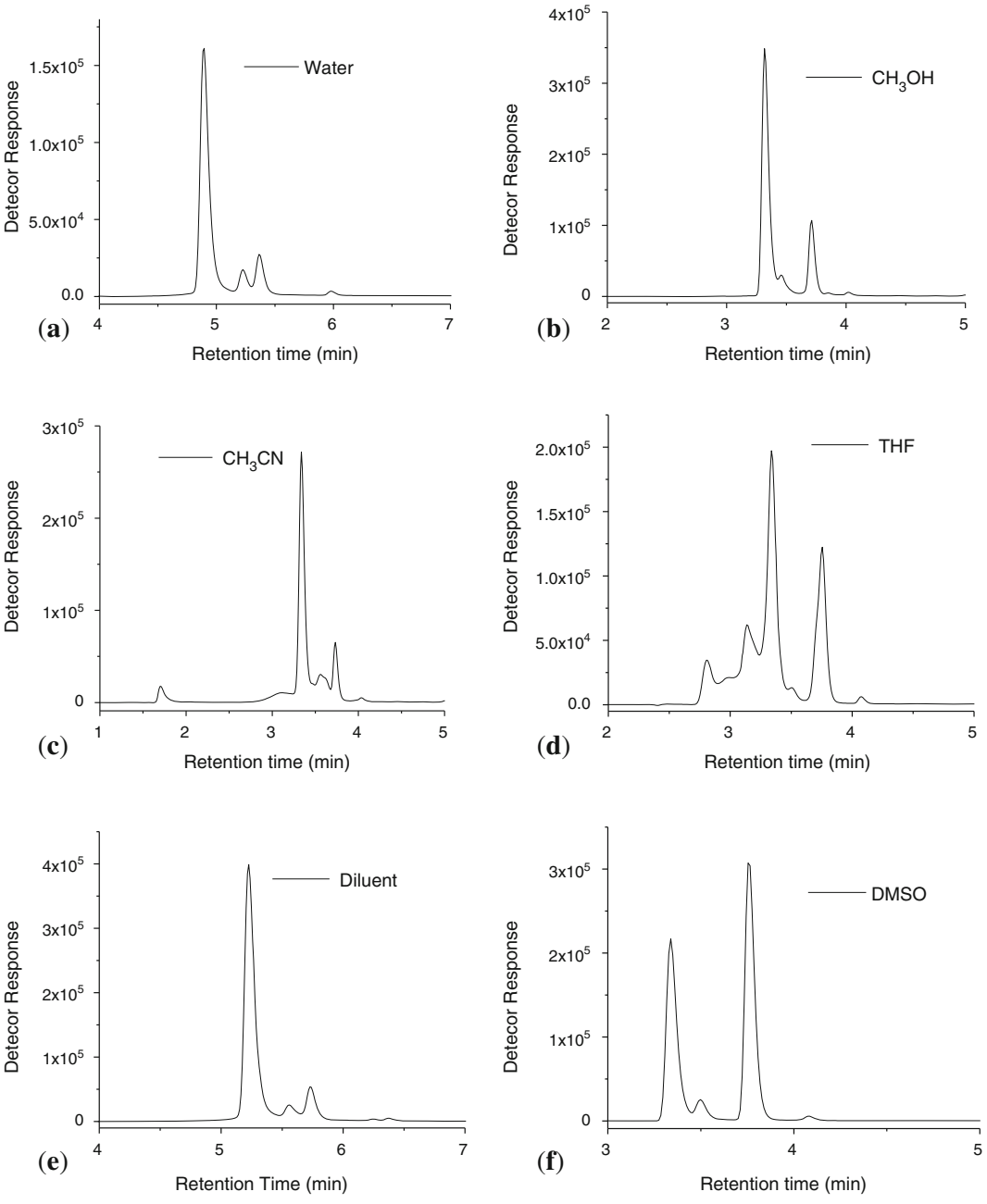


Figure 5. Effect of solvent on retention and separation of isomers of Phox; (**a**) H_2O, (**b**) CH_3OH, (**c**) CH_3CN, (**d**) THF, (**e**) diluent, and (**f**) DMSO.

conditions (method IV) have been used and chromatograms obtained are shown in figure 3. Retention time and the percentage of isomers depends on the observed as ~50:50 in water and diluent, this may be due to nearly same stability of both isomers in water, ~60:40 in methanol and ~30:70 in DMSO. Peak purity index observed to be ~1 hence there is no merged impurity in both the peaks. More than two peaks are observed in chromatograms of THF and CH_3CN solvents, this may be due to the instability of tautomers in these solvents or presence of *syn* and *anti* stereoisomer's. Thus, separation of Lwox in various solvent gives us an idea about the presence of tautomers instead of stereoisomers (table 2).

3.8 *Separation of 2-hydroxy-3-methy-4-naphthoquinone-1-oxime (Phox) isomers*

Separation of Phox isomers have been attempted in various pH of the mobile phase. The desired pH is achieved using appropriate buffer solutions. Figure 4 shows chromatogram for Phox separation obtained at pH 7, 4.5, 6.8, 8.5, 10.5 and isocratic separation and chromatographic data are presented in table S2.

(i) At pH 7 (without modifier) Phox retain on column and there is no interaction of Phox with mobile phase and no separation of the peaks are observed. (ii) At pH 2.5 (figure S5), there are three peaks were observed, two peaks are of isomers at Rt-5.570 min and Rt-5.804 min with mass 204 (M + 1) and a peak due to impurity with *m/z* 201 [M + 1]. The percentage of impurity is significantly less. The separation of isomer is not base to base, however the impurity is well-separated from the desired isomers. (iii) At pH 4.5, resolution between isomers is observed, however there is no base to base separation. Three peaks are observed, two of the peaks belong to isomers while one peak due to impurity. The unequal concentration of isomers is observed at this pH. Phox retains on column. (iv) At pH 6.8, the impurity peak (Rt-3.750 min) is merged in isomer X′ (Rt-3.968) and there is no separation between the isomers. (v) At pH 8.5, separation between the isomer peaks is 0.6 min.

Table 3. Chromatographic data obtained of peaks of figure 5a–f at 254 nm.

Sl. No.	Solvent	Ret. time (in min)	% Area	Peak purity index	Tailing factor
1.	H_2O	4.887	84.27	1.000	1.64
		5.222	04.99	1.000	1.16
		5.362	09.43	0.990	1.44
		5.974	01.11	0.999	1.37
2.	CH_3OH	3.314	74.54	0.949	1.47
		3.453	01.27	0.591	1.60
		3.705	21.11	0.999	1.37
		4.014	00.72	0.999	1.48
3.	Diluent	5.220	88.68	1.000	1.59
		5.552	02.67	0.974	1.33
		5.726	08.07	1.000	1.44
		6.367	00.56	0.999	1.51
4.	DMSO	3.333	41.51	0.968	1.66
		3.493	04.50	0.977	–
		3.754	53.14	0.999	1.52
		4.075	00.84	0.999	1.19
5.	THF	2.804	07.44	1.000	–
		2.984	06.65	0.992	–
		3.135	18.99	0.999	–
		3.335	41.04	0.939	–
		3.498	01.45	0.990	–
		3.748	23.65	1.000	0.98
		4.069	00.75	1.000	1.38
6.	CH_3CN	1.699	04.61	0.143	–
		3.105	7.560	0.945	–
		3.334	62.53	0.694	–
		3.556	12.80	0.793	–
		3.727	11.62	0.472	–
		4.031	00.86	1.000	–

Table 4. Effect of treatment of **3** and **4** in three different cell lines.

	COLO 205	U87 MG	MIA PaCa-2
3	Resistant	Inactive	Inactive
4	Resistant	Inactive	Inactive

Isomer X′ (Rt-3.443 min) shows a tailing effect, which presumably arises from a merged impurity (tailing factor 0.953). An impurity peak is observed (Im) at (Rt-4.397 min), it is separated from isomer Y′. (vi) At pH 10.5, four peaks are observed. Two peaks belong to impurity. Impurity peaks are confirmed by LC-MS analysis with *m/z* 201 [M + 1]. (vii) Broad peaks are observed in isocratic elution, thus the isocratic elution is not suitable for the separation and elution of isomers.

3.9 *Effect of solvent polarity on separation of the isomers of Phox*

A neutral chromatographic condition (method IV) is used for the study of the effect of solvent on the separation of isomers/tautomers of Phox. Retention time and the percentage of isomer depend upon the polarity of the solvents as shown in figure 5a–f; the chromatographic data is presented in table 3. Four peaks are observed in water, methanol and diluent solvents, the percentage of isomer X′ is more than the isomer Y′, however the percentages of isomers varies with solvent polarity. In polar protic solvent, the isomers retained on the column may be due to '*inter*' or '*intra*' molecular hydrogen bonding. But the peaks were not separated base to base in DMSO, THF and CH_3CN solvent; hence these solvents are not suitable for the elution of Phox.

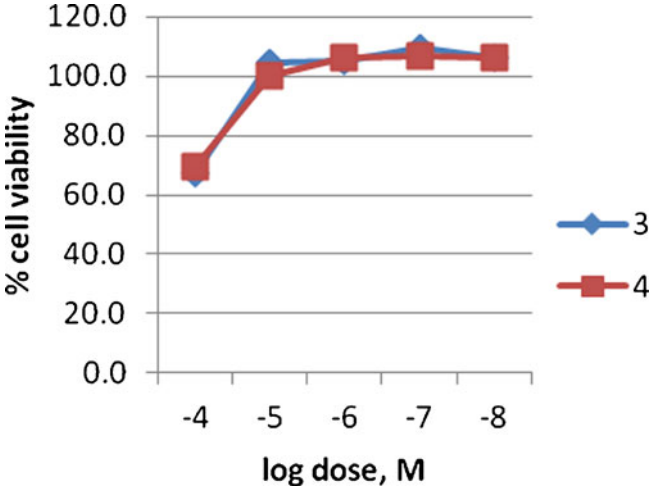


Figure 6. Dose-response curve upon treatment of COLO 205 cell line with **3** and **4**.

3.10 *In vitro cell viability assay of tautomers **3** and **4** of Lwox*

Cell viability studies on three cancer cell lines of different tissue origin upon treatment with **3** and **4** at varying concentration using XTT assay were studied. From the results it was observed that tautomers **3** and **4** were inactive in cell lines (table 4) U87 MG and MIA PaCa-2 at concentration > 10 mM. COLO 205 cell line treated with 10 mM concentration of compounds resulted in 67% and 69% of viable cells (figure 6). However, doxorubicin was active in all **3** cell lines with an IC_{50} value of 0.68 nM, 0.34 μM and 0.1 nM in COLO 205, U87 MG and MIA PaCa-2 cell lines, respectively (figure 7).

3.11 *DFT calculations*

Geometry-optimized structures for tautomers **3** and **4** (**3**′ and **4**′ in Phox) were computed using the B3LYP method. The optimized structures are presented in figure 8 for Lwox tautomers and in figure 9 for Phox tautomers. Structures **3** and **3**′ are 4.7 and 5.8 kcal mol^{-1} more stabilized than **4** and **4**′, respectively, as a result of a hydrogen bonding interaction between the 2-hydroxyl group and the nitrogen of the oxime. This affords the favourable *amphi* disposition of the N–OH group leading to a short contact of 2.131 Å in Lwox and 2.129 Å in Phox with nearby H(8) of the aromatic ring. The C(1)–N(1) and C(4)–O(4) distances of 1.290 Å and 1.235 Å in Lwox, and 1.290 Å and 1.234 Å in Phox, respectively are in the range for a double bond; the C(2)–O(2) distance of 1.338 Å and 1.342 Å in Lwox and Phox is consistent with single bond. Swapping the hydroxyl and ketyl groups in **4** results in a slight shortening of the N–O· · ·H(8) interaction by ∼0.04 Å concomitant

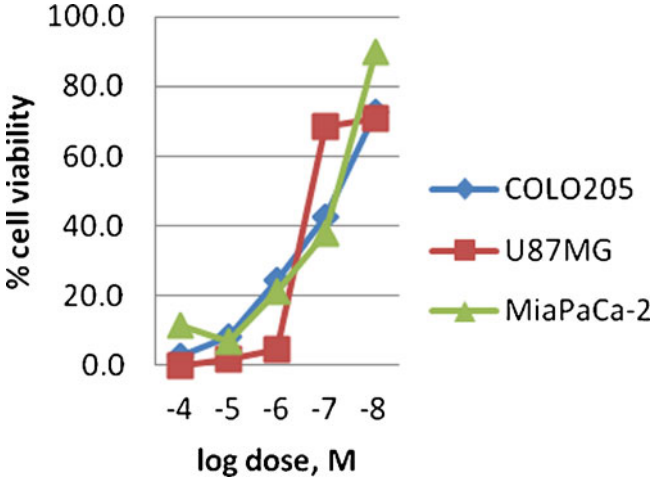


Figure 7. Dose-response curve upon treatment with doxorubicin in all three cell lines (COLO 205, U87 MG and MIA PaCa-2).

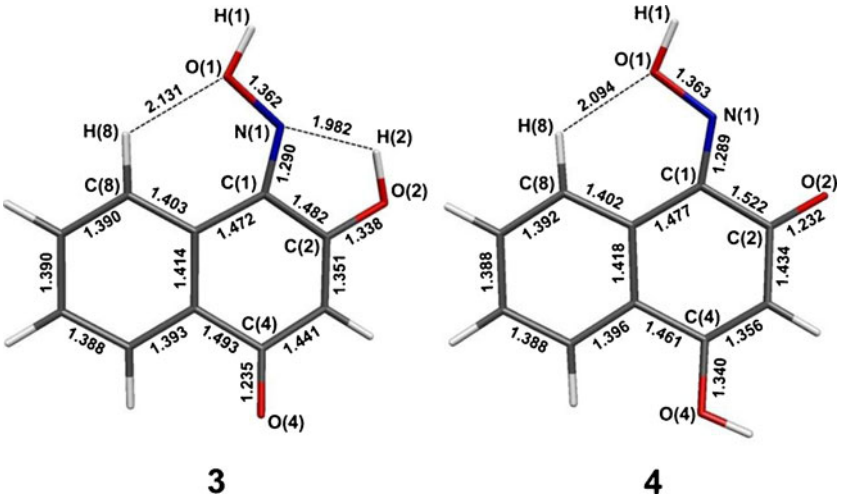


Figure 8. Geometry optimized structures for **3** and **4** from B3LYP-DFT calculations. Indicated distances are in Å.

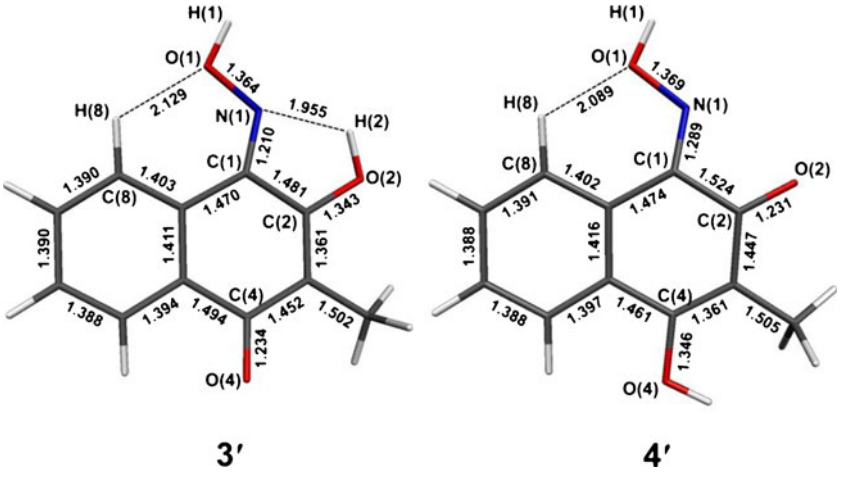


Figure 9. Geometry optimized structures for **3′** and **4′** from B3LYP-DFT calculations. Indicated distances are in Å.

with the absence of any intramolecular hydrogen bonding with the oxime nitrogen atom, no energy regained via new interactions with the 4-hydroxyl substituent due to the absence of suitable donor groups in its vicinity.

4. Conclusions

The separation, isolation and characterization of tautomers of naphthoquinoneoxime have been carried out for the first time. The influence of pH on the mobile phase has been studied on the separation of isomers of 2-hydroxy-4-naphthoquinone-1-oxime; Lwox and 3-methyl-2-hydroxy-4-naphthoquinone-1-oxime; Phox. The pH has been maintained using various buffer solutions in water. Two distinct peaks are observed in pH range studied between pH 2.5 and 10.5 for Lwox. Tautomeric equilibrium has been observed in acidic pH of mobile phase, while base to base separation of the isomers is present at neutral and basic pH of mobile phase in Lwox. Tautomers **3** (2-hydroxy-4-naphthoquinone-1-oxime) and **4** (4-hydroxy-2-naphthoquinone-1-oxime) are isolated in solid state by preparative HPLC technique and are characterized by LC-MS, FT-IR, and UV-visible spectra. Tautomers **3** and **4** are stable in solid state, while their fast interconversion to one another is observed in solution. FT-IR spectra of **3** show distinct paranaphthoquinone frequency at ∼1280–1290 cm^{-1}, this frequency is absent in **4**. Charge transfer bands are observed in UV-visible spectra at 420 nm and 405 nm for red orange, **3** and yellow, **4**, respectively.

Separation of isomers/tautomers of Phox cannot be achieved either by chromatographic methods used nor

by varying polarity of solvents. There could be two reasons for the same, (a) chromatographic conditions are inadequate for separation and (b) presence of stereoisomers such as *syn* and *anti* as well as *ortho* and *para* tautomers present in solution.

Cell viability studies on three cell lines showed that **3** and **4** were inactive in both U87MG (glioblastoma astrocytoma) and MIAPaCa-2 (human pancreatic carcinoma) cancer cell lines some degree of sensitization was observed in COLO205 (human colorectal adenocarcinoma). In the contrary, doxorubicin, which was used as a positive control was very active in all tissue types at a nanomolar range. From these studies it may be concluded that **3** and **4** can be used as potential lead compounds to specifically target against colon carcinoma without cross reactivity against any other vital tissues.

Geometry-optimized structures for tautomers **3** and **4** (**3′** and **4′** in Phox) were computed using the B3LYP method. Structures **3** and **3′** are 4.7 and 5.8 kcal mol^{-1} more stabilized than **4** and **4′**, respectively, as a result of a hydrogen bond interaction between the 2-hydroxyl group and the nitrogen of the oxime.

Supplementary information

Details of experimental conditions for separation of isomers by HPLC, methods I to IX, figures S1–S5 and tables S1 and S2 can be seen at www.ias.ac.in/chemsci website as supplementary information.

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