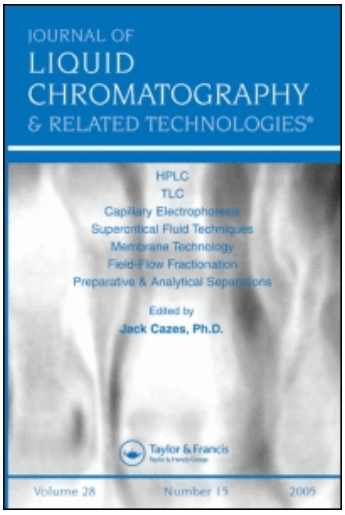

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HPLC Separation of Tautomeric Compounds of 4-Aminoisoxazolyl-1,2-naphthoquinones. II

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HPLC SEPARATION OF TAUTOMERIC COMPOUNDS OF 4-AMINOISOXAZOLYL-1,2-NAPHTHOQUINONES. II

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ABSTRACT

HPLC separations and quantitative analysis are described for a mixture of 4-aminoisoxazolyl-1,2-naphthoquinone isomers. This assay is simple, rapid and stability indicating because the precursors and isomerization products can be monitored simultaneously. The results obtained are in agreement with those obtained by UV spectroscopy.

INTRODUCTION

Previous results from our laboratory[1] have indicated the usefulness of the HPLC technique in the separation and analysis of substances with only minor structural differences as in the case of keto and enol tautomers of some isoxazolyl-naphthoquinones.

As an extension of this work, we decided to investigate the applicability of HPLC procedure for

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the determination of six new methylated isoxazolyl-naphthoquinones: N-(5-methyl-3-isoxazolyl)-1,2-naphthoquinone-4-amine (I); 2-hidroxi-N-(5-methyl-3-isoxazolyl)-1,4-imine (II); N-(3-methyl-5-isoxazolyl)-1,2-naphthoquinone-4-amine (III); 2-hidroxi-N-(3-methyl-5-isoxazolyl)-1,2-naphthoquinone-4-imine (IV); 2-methoxy-N-(5-methyl-3-isoxazolyl)-1,4-naphthoquinone-4-imine (V) and 2-methoxy-N-(3-methyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine (VI).

These compounds,which were prepared by a procedure previously described, offer the interesting possibilities of examining the effects of structure on the stability of the tautomeric system.

EXPERIMENTAL

Instrumentation

The analytical HPLC work was performed on a Schimadzu liquid chromatograph equipped with LC-2A pumping system and SPD-2A UV detector operated at 330 nm. The detector was connected to an Altex CR_1 integrator. The column was operated at room temperature. The compounds were eluted isocratically at a pressure of about 1.200 psi.

The optimun values of HPLC parameters are shown in table N°1.

TABLE 1

The Optimun Values Of The HPLC Parameters

Volume Injected	20 µl
Column	Analytical prepacked 5 micron Silica Gel from Micromeritics.
Mobile Phase	Methanol-Chloroform (20:80).
Flow Rate	1.2 ml/min.
Detector	330 nm.
Sensitivity	0.02 Amps.
Chart Speed	o.5 cm/min.

The examined samples were dissolved in ethanol and injected via loop-injector.

M.p. were determined by the capillary method on a Buchi apparatus and are uncorrected.

Thin layer chromatograms were carried out on silica gel plates using benzene, chloroform and ethanol (10:30:4) as developing system. Column chromatography was performed on silica gel (Merck).

REAGENTS AND MATERIALS

Chloroform was analytical grade and was distilled prior to use. Methanol and ethanol were treated with 2,4-dinitrophenylhydrazine according to reference[2].

Compounds I, II and V were synthesized and purified as previously reported[3]. Compounds III and IV were synthesized by the reaction of the sodium salt of 1,2-naphthoquinone-4-sulfonic acid with 3-methyl-5-aminoisoxazole[4]. Methylation of IV with diazomethane afforded VI.

The structures of these compounds were determined by UV, IR, ^{1}H-NMR, MS and by elemental analysis.

Standard And Reference Solutions

The stock solutions of the compounds I-IV were prepared by dissolving 0.030 mg in 100 ml of ethanol.

Reference standard solutions were also prepared in ethanol by dissolving compounds V and VI to a concentration similar to that of standard solutions.

Calibration Curve

A 0.3 μg/ml ethanol solution of every compound was diluted with the solvent to obtain five standard solutions ranging from $1.2\text{-}3.10^{-2}$ μg/ml.

Triplicate 20 μl aliquots of working volume sample and working reference standard solutions were alternately injected into the liquid chromatograph.

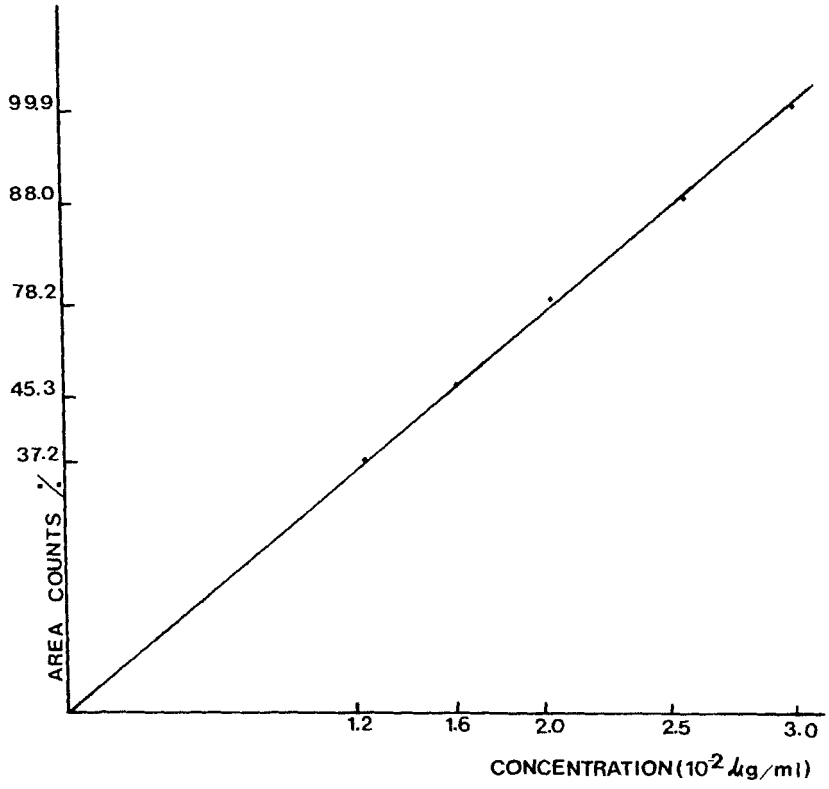


Figure 1: Compound II standard curve.
Each point is the average of 3 determination.

Quantification of the samples was carried out by the Altex CR 1-A Data System. Figure 1 shows the standard curve which was obtained by plotting area count (%) Vs. concentration of II. The validity of the calibration graph was checked daily.

Standard and reference solutions were simultaneously determined by UV spectroscopy[5], using light of 330 nm as the analytical wavelength.

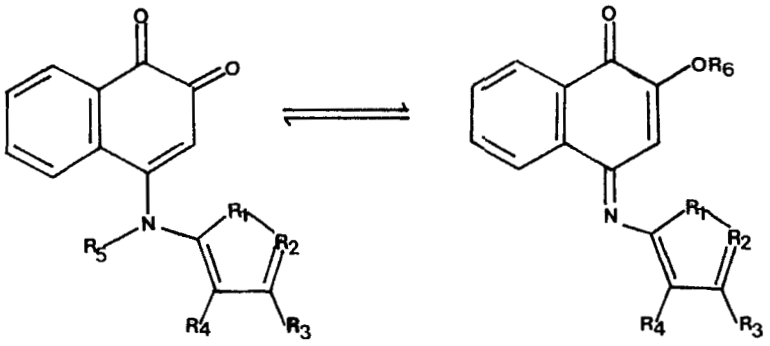
RESULTS AND DISCUSSION

Similarly as in the previous paper[1] the chromatographic and spectroscopic properties of this family of compounds have proved to be useful for their separation and analysis by HPLC with ultraviolet detection using commercially available silica gel column.

These compounds (Table 2) needed a mobile phase with a high content of chloroform. The mixture methanol-chloroform (20:80) revealed to be the most successful of the systems investigated since it permitted the resolution of the tautomers in samples arising from the reaction mixture, the pure form (figure 2, table 3) as well as in combination (figure 3, table 4).

TABLE 2

Structure Of 4-Aminoisixazolyl-1,2-Naphthoquinones



Compound	R_1	R_2	R_3	R_4	R_5	R_6
I	N	O	CH_3	H	H	-
II	N	O	CH_3	H	-	H
III	O	N	CH_3	H	H	-
IV	O	N	CH_3	H	-	H
V	N	O	CH_3	H	-	CH_3
VI	O	N	CH_3	H	-	CH_3

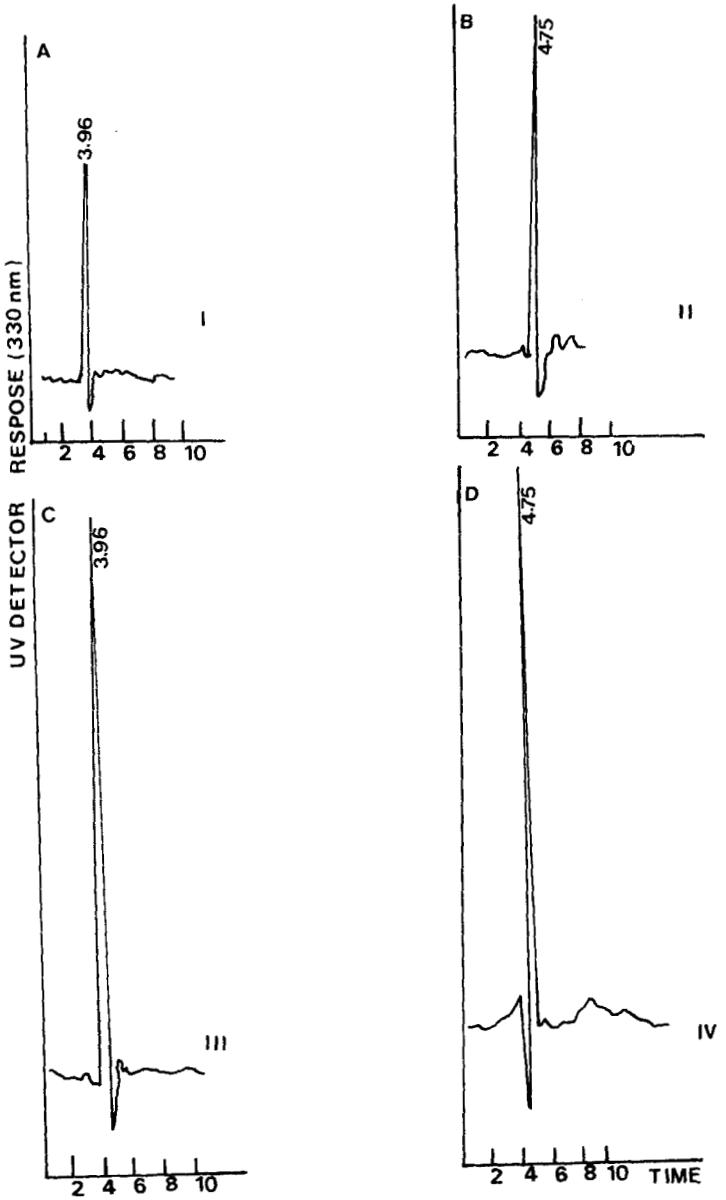


Figure 2: HPLC-UV chromatograms of st. samples of isoxazolylnaphthoquinones.
A: compound I; B: compound II; C: compound III and D: compound IV.

TABLE 3

Recovery Of Isoxazolyl-Naphthoquinones From Ethanolic Solutions By HPLC.

Sample	% Recovery			Retention time (min.)		
	K	E	OCH_3	K	E	OCH_3
I	100	-	-	3,96	-	-
II	-	100	-	-	4,75	-
III	100	-	-	3,96	-	-
IV	-	100	-	-	4,75	-
V	-	-	100	-	-	4,84
VI	-	-	100	-	-	4,89

TABLE 4

Recovery Of Isoxazolyl-Naphthoquinones From Standard Mixtures

Sample	% Recovery		Retention time (min.)	
	K	E	K	E
I + II (70:30)	67	33	3.96	4.75
I + II (50:50)	55	45	3.96	4.75
I + II (30:70)	29	71	3.96	4.75
III + IV (70:30)	62	37	3.96	4.75
III + IV (50:50)	51	49	3.96	4.75
III + IV (30:70)	22	78	3.96	4.75

As seen by the retention time given in tables 3 and 4, there is a good separation of the tautomers. The retention time values for II and IV are the same irrespective of the site of the substituting methyl group in the isoxazole molecule. The same situation is observed in the case of the keto forms I and III and for the methyl derivatives V and VI.

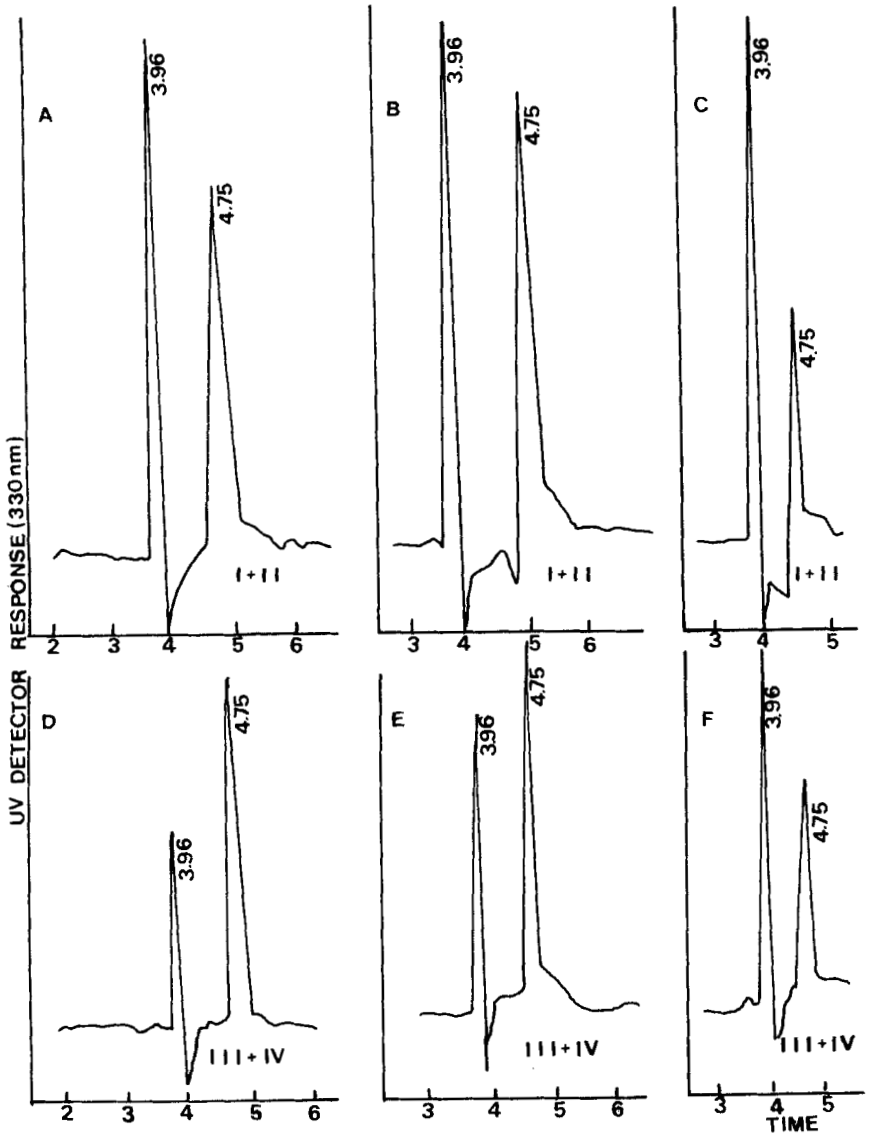


Figure 3: HPLC-UV Chromatograms of standard samples of isoxazolyl-naphthoquinones.
A, B and C: mixtures of I + II, 70:30; 50:50; 30:70, respectively; D,E and F: mixtures of III + IV, 70:30; 50:50; 30:70, respectively.

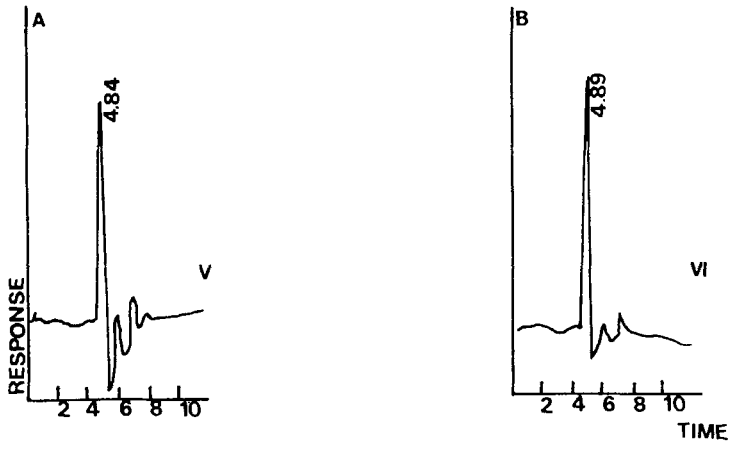


Figure 4: HPLC chromatograms of reference standard; A: compound V and B: compound VI.

The O-methylated fixed tautomers V and VI (figure 4) were used as external standards for the pairs I-II and III-IV respectively.

In order to assure that the elution of the compounds was complete for each series of HPLC measurements one or two samples were analyzed up to 30 minutes.

The chromatographic capacity factors, K', were calculated by equation

$$K' = \frac{t - t_o}{t_o}$$

where t is the retention time of the compounds of interest and t_o is the retention time of the inert solvent. They reveal the minimum value corresponding to the keto form.

Chromatographic resolution was calculated by equation 2

$$Rs = \frac{(t_2 - t_1)}{1/2(W_2 + W_1)} = 1.45$$

where t_1 and t_2 are the retention times of the keto and enol forms, and W_1, W_2 are the widths of the two peaks at their half-heights[6].

Linearity of the method with respect to concentration of compounds I-IV was checked. Peak areas were used to construct calibration curves giving a correlation coefficient of 0.99.

Replicate and triplicate measurements showed that the values for retention time and for porcentage amount varied in a range of ± 2%.

The accuracy of the method was investigated for compounds I and III using V and VI as internal standards.

Calibration mixtures containing different amounts of each tautomer are expressed in table 4. The ratio of peak areas was used to calculate a theoretical concentration.

Stability Studies

The effect of storage on the stability of compounds I-IV in ethanolic solutions was analyzed.

These isomers can be distinguished from each other by column, TLC and by proton NMR spectroscopy[3], and their physical properties were determined without difficulty.

In the solid state they are stable at room temperature, and they can be differentiated by IR spectroscopy[4] on the basis of the C=O group vibrations.

The results previously obtained using column chromatography as the analytical method were different from those obtained by ^{1}H-NMR spectroscopy[4], probably due to the basicity of the solvent used (DMSO).

The development of this HPLC method allowed us not only to know with accuracy and precision the composition of the tautomeric mixtures but also to determine the relative stability of both tautomeric systems and that of each of the products formed in the reaction medium.

The data in table 3 were obtained with ethanolic solutions recently prepared. They show that all of the compunds are stable in those conditions.

However, these results undergo variation with the time. An experiment made with compound II yielded 21% of the keto and 79% of the enol form 1 hour after the solution was prepared.

These changes observed were still more important after 24 hours, as we can see in table 5.

The determined amounts indicated that the stability of the tautomeric systems is different for both pairs of tautomers. In the system I-II the keto form always predominates in ethanolic solution, whereas for the system III-IV the enol form is favoured.

TABLE 5

Time Effect On Tautomeric Equilibria Of Isoxazolyl-Naphthoquinones. Ethanolic Solutions After 24 Hours.

Sample	% Recovery HPLC		Retention time (min.)	
	K	E	K	E
I+II (70:30)	81,6	18	3,96	4,75
I+II (50:50)	53	46	3,96	4,75
I+II (30:70)	68	32	3,96	4,75
III+IV (70:30)	40	60	3,96	4,75
III+IV (50:50)	42	58	3,96	4,75
III+IV (30:70)	14	86	3,96	4,75

These results are consistent with those previously obtained[3,4], indicating that the rate of tautomeric change depends principally on the structure of the isoxazole as well as on the polarity of the solvent and is not affected by the absorbent used (silica gel) in the condition specified for the analysis.

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