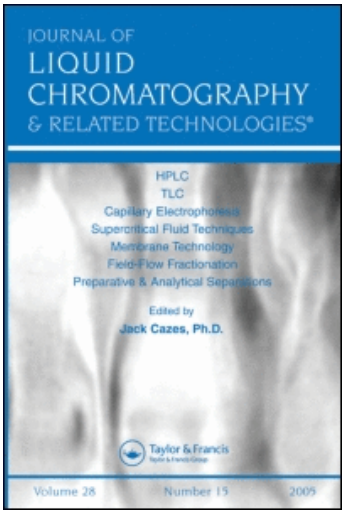

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HPLC SEPARATION OF TAUTOMERIC COMPOUNDS OF 4-AMINOISOXAZOLYL-1,2-NAPHTHOQUINONES

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ABSTRACT

A sensitive and efficient method for determining a mixture of 4-aminoisoxazolyl-1,2-naphthoquinones isomers was developed using high performance liquid chromatography. The assay is also stability indicating because precursors and isomerization products can be determined simultaneously.The results obtained are in agreement with those obtained by UV spectroscopy.

INTRODUCTION

Several methods have been described for the determination of tautomeric compounds. These include potentiometric[1], nonaqueous titrations and direct spectrometric measurement[2,3].

During the synthesis of new 4-aminoisoxazolyl-1,2-naphthoquinones[4], we verify the formation of a mixture of tautomeric compounds, which were separated by TLC and column chromatography.

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We have also developed a quantitative method for the analysis of tautomeric compounds by ^{1}H-RMN[5] in dimethyl sulfoxide. It was apparent that the values obtained by this method were considerably different from those obtained by column chromatography.

An analytical procedure was then required for their determination in samples arising from the reaction mixture and from the pure form. This procedure had to be accurate and precise, specific in the presence of isomerization products, rapid, simple and stability indicating.

This paper reports the development of a simple, rapid precise and specific HPLC procedure for the determination of N-(3,4-dimethyl-5-isoxazolyl)-1,2-naphthoquinone-4-amino (I); 2-hidroxi-N-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine (II); N-(4-methyl-5-isoxazolyl)1,2-naphthoquinone-4-amino (III) and 2-hidroxi-N-(4-methyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine (IV), which were previously synthetized[4] by reaction between the sodium salt of 1,2-naphthoquinone-4-sulfonic acid and the corresponding amino-methylisoxazole.

EXPERIMENTAL

Instrumentation

A constant volume liquid chromatograph from Beckman, consisting of a model 110 A Solvent delivery

TABLE 1

The Optimum Values of the HPLC Parameters

Volume inyected	25 ul
Column	Analytical prepacked 5 micron Silica Gel from Micromeritics
Mobile phase	Methanol-chloroform (90:10)
Flow rate	1,4 ml/min.
Detector	330 nm
Sensitivity	0,02 Amps.
Chart Speed	0,5 cm/min.

system and a Hitachi UV-Vis detector (interfaced with a Altex CR1 A Data System) set at 330 nm, was used.

The optimum values of the established HPLC parameters are shown in Table 1.

UV spectra were carried out in a Beckman DB-G spectrophotometer using 1 cm quarz cells.

Reagents and Materials

Benzene and chloroform were analytical grade and were distilled prior to use. Methanol was treated with 2,4-dinitrophenylhydrazine according to ref.[6].

The isoxazolyl-naphthoquinones were synthesized in these laboratories in the same way as their has been previously reported[4]. 2-Methoxy-N-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine (V) and 2-methoxy-N-(4-methyl-5-isoxazolyl)-1,4-naphthoquinone-

4-imine (VI), were used as internal standard, and they were prepared by reaction between II and IV with diazomethane[4].

Standard and Reference Solutions

The stock solutions of the purified compounds (I-IV) were prepared in distilled water or ethanol by dissolving 15 mg in 100 ml of solvent.

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

Calibration Curve

A 0,150 mg/ml aqueous or ethanol solution of every compound was diluted with the appropiate solvent to obtain five standard solutions ranging from 2 to 14 ug/ml.

Triplicate 25 ul aliquots of working volume samples and working reference standard solutions were alternately injected into the liquid chromatograph.

Quantification of the samples was carried out by the Altex CR1-A Data System. Calculations were performed using the peak height calibration mode.

Figure 1 shows a standard curve which was obtained by plotting area count (%) vs concentration of II.

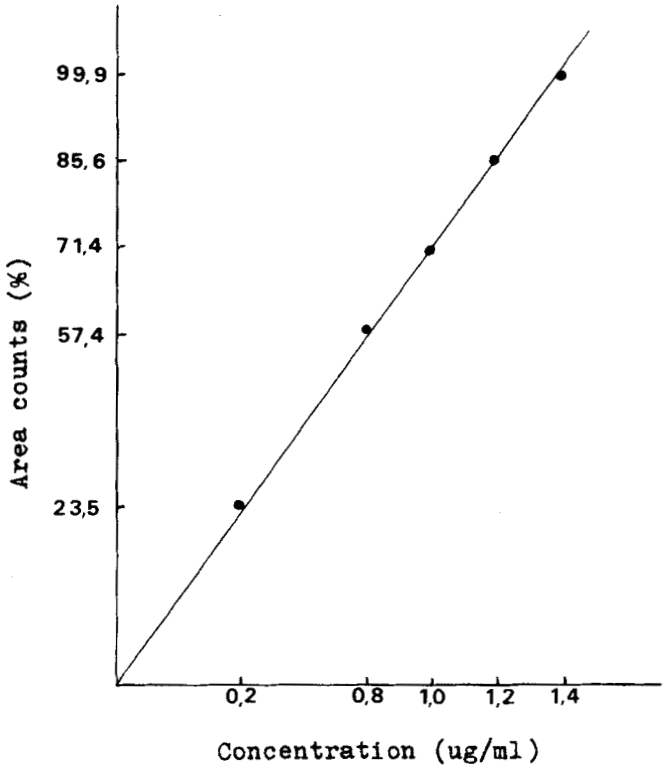


Figure 1: Compound II standard curve. Each point is the average of 3 determination.

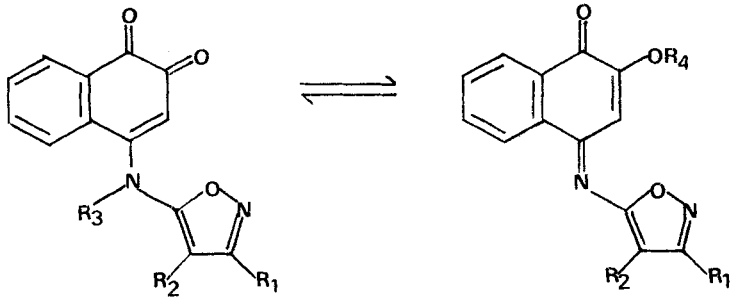
Standard and reference solutions were simultaneously determined by UV spectroscopy[5], using light of 330 nm as the analytical wavelength.

RESULTS AND DISCUSSION

The UV absorption spectra and the chromatographic properties of these drugs have proved to be useful for their separation by HPLC with ultraviolet detection.

TABLE 2

Structure Of 4-Aminoisoxazolyl-1,2-Naphthoquinones



Compound	Subst.R_1	Subst.R_2	Subst.R_3	Subst.R_4
I	CH_3	CH_3	H	---
II	CH_3	CH_3	---	H
III	H	CH_3	H	---
IV	H	CH_3	---	H
V	CH_3	CH_3	---	CH_3
VI	H	CH_3	---	CH_3

The compounds (Table 2), as single entities as well as in combination (Figure 2, 3) were analyzed succesfully using normal silicagel column HPLC.

Two different mobile phases were used: benzene-acetonitrile-chloroform (10:30:10) and chloroform-methanol (10-90). The best results were obtained with the second mobile phase.

Linearity of the method with respect to concentration of compounds I-IV was checked. Peak areas were used to construct calibration lines giving a correlation coefficient of 0,998.

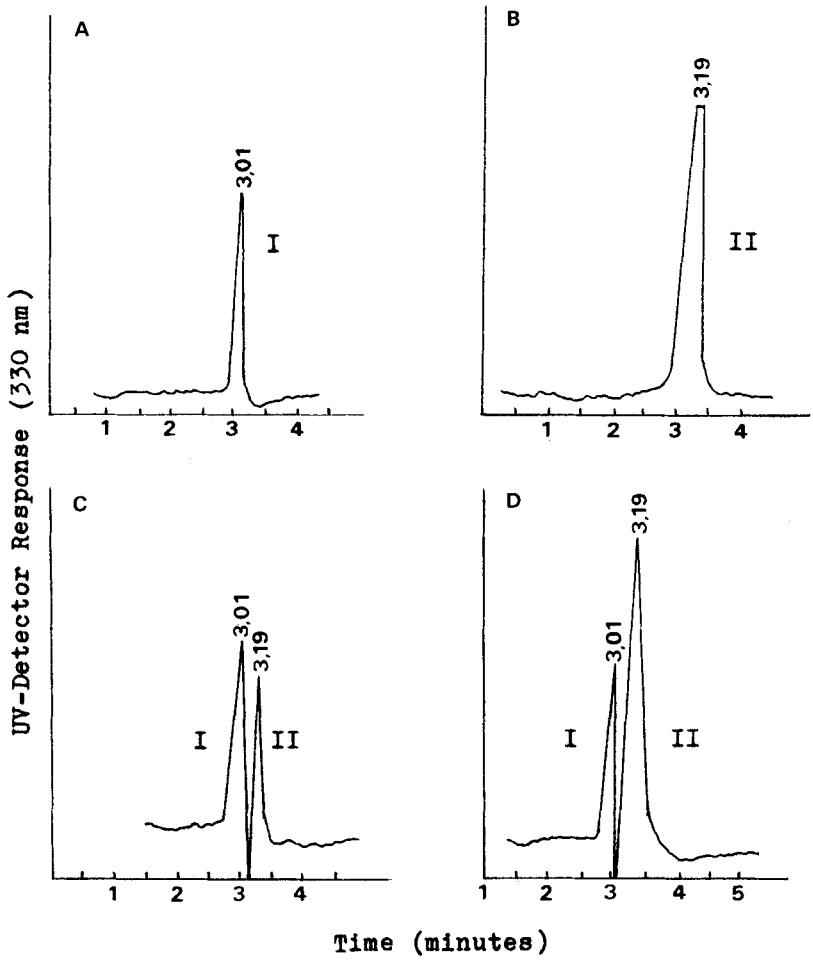


Figure 2: HPLC-UV Chromatograms of standard samples of isoxazolyl-naphthoquinones. A: compound I; B: compound II; C and D mixtrues of I + II, 50:50 and 25:75, respectively.

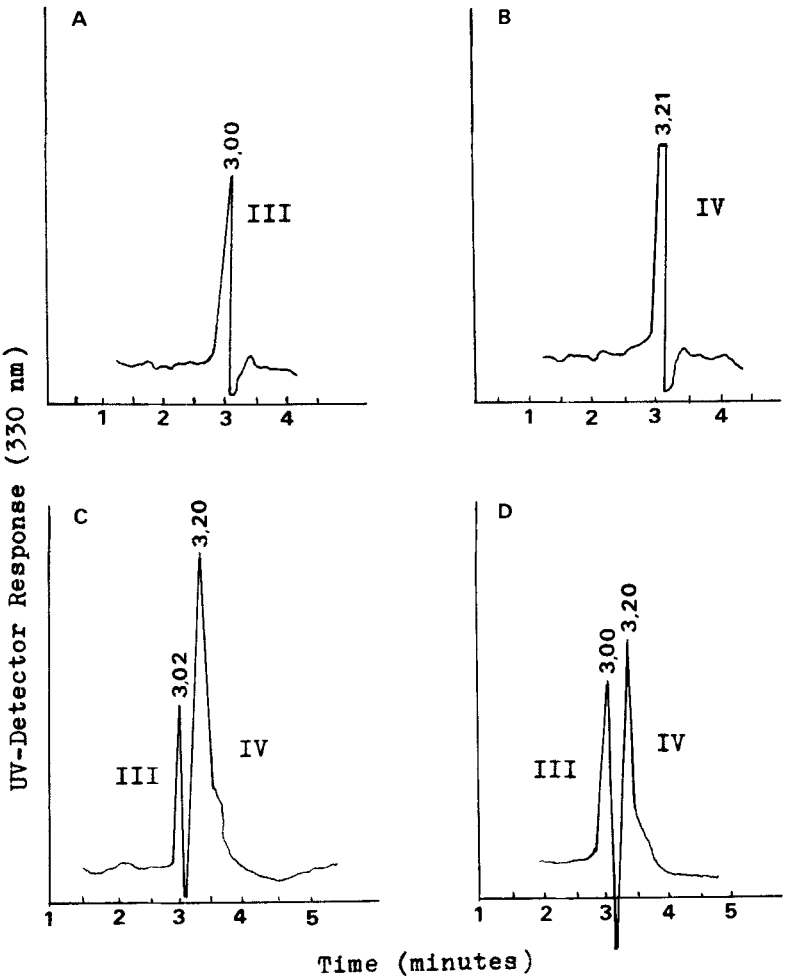


Figure 3: HPLC-UV chromatograms of standard samples of isoxazolyl-naphthoquinones. A: compound III; B: compound IV; C and D: mixtures of III + IV, 20:80; 40:60, respectively.

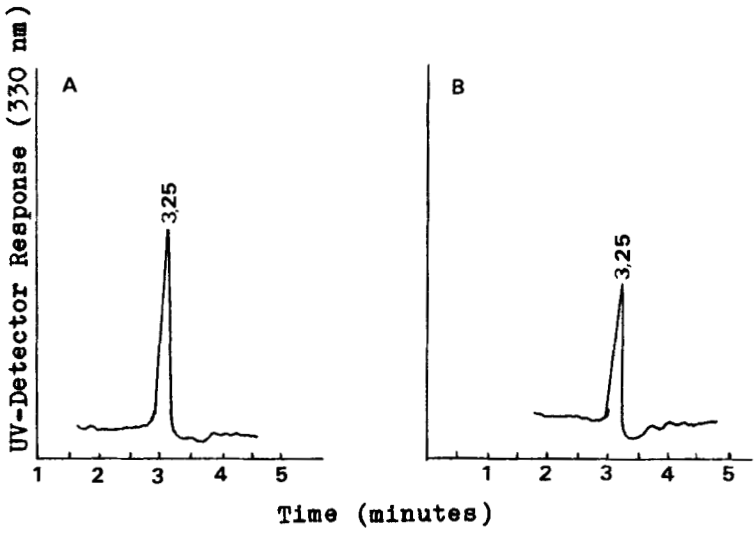


Figure 4: HPLC chromatograms of reference standard; A: compound V and B: compound VI.

Reproducibility of three 25 ul repeated injections of each solution for the compounds II, IV and V was established and the relative Standard deviations of the count/weight ratios were 0,2; 0,4 and 0,5 respectively.

The accuracy of the method was investigated for compounds II and IV using V and VI (Figure 4) as internal standards.

The ratio of peak areas was used to calculate a theoretical concentration. The results were compared with those of the UV assay and are shown in Table 3 and as it can be seen, most of them are in good agreement.

TABLE 3

Recovery Of Isoxazolyl-Naphthoquinones From Ethanolic Solutions By UV And HPLC

Sample	% Recovery		Retention time min.
	UV	HPLC	
I	97,0	100 ± 0,5	3 - 3,01
II	99,9	100 ± 0,2	3,19 - 3,21
I + II	50:50	56,55 ± 43,4	3 - 3,19
I + II	25:75	22 - 77	3,02 - 3,18
III	96	100 ± 0,5	3 - 3,02
IV	89	100 ± 0,4	3,21 - 3,19
III + IV	20:80	17 - 83	3,02 - 3,21
III + IV	40:60	40 - 61	3,02 - 3,21
V	99	100	3,25
VI	99	100	3,25

Stability Studies

The effect of storage on the stability of compounds I-IV in ethanolic solutions and in aqueous acidic and basic solutions was also analyzed.

The results of the isomerization of these compounds at room temperature as measured at 24 hs after their preparation are shown in Table 4.

The isomerization of II and IV in ethanol to give I and III respectively, was undetected over the 24 hs

TABLE 4

Medium Effect On Tautomeric Equilibria Of Isoxazolyl-Naphthoquinones

Sample	% Recovery UV	% Recovery HPLC	Retention Time min.	Figure
I[b]	75-25 K E	70-30	3,03-3,73	7
II[a]	70 K	85-12-2 K E	3,03-3,73-4,02	5
IV[a]	65 K	75-10-1 K E	3,02-3,80-4,02	5
I[c]	---	33-66 K E	3,03-3,19	6
III[c]	---	16-83 K E	2,96-3,19	6

a: aqueous acidic solution; b: Aqueous basic solution; c: ethanolic solution; K: ketoform; E: enolic form.

period. However, their solutions in aqueous acidic medium reveal, after 24 hs, their conversion to the keto form (Figure 5).

The behavior of I and III is different from that of II and IV. Both compounds in ethanolic slutions, after 24 hs, changed into their enolic form (Figure 6).

HPLC analysis of I in aqueous basic medium, indicated that the solution analyzed was a mixture of

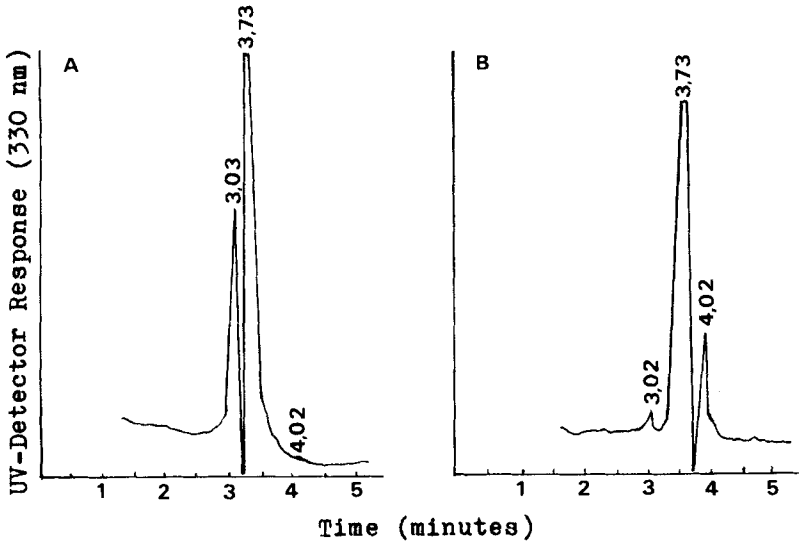


Figure 5: Chromatograms of an aqueous acidic solutions of isoxazolyl-naphthoquinones after 24 hs. A: compound II and B: compound IV.

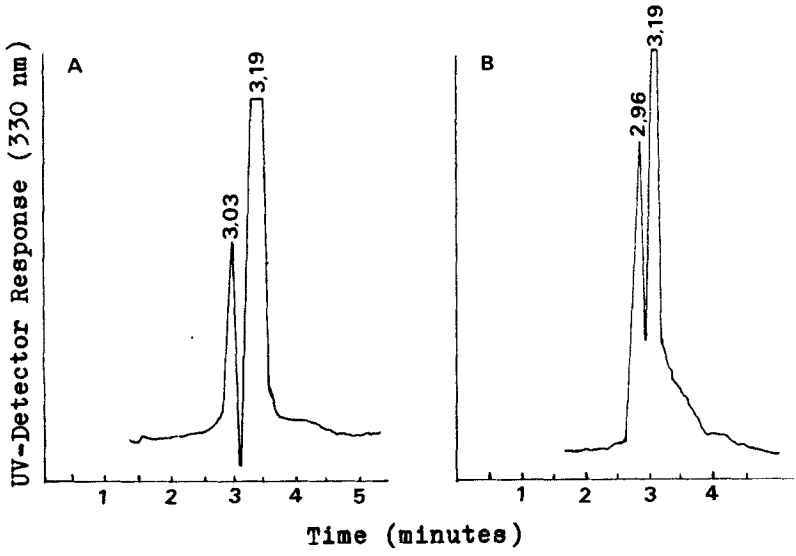


Figure 6: Chromatograms of an ethanolic solutions of isoxazolyl-naphthoquinones after 24 hs. A: compound I; B: compound III.

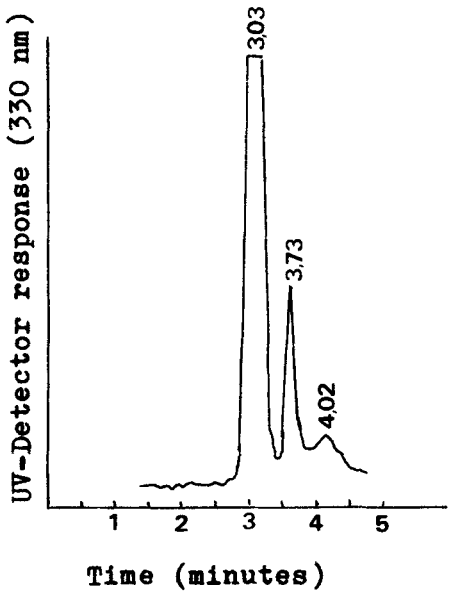


Figure 7: Chromatogram of an aqueous basic solution of I, after 24 hs.

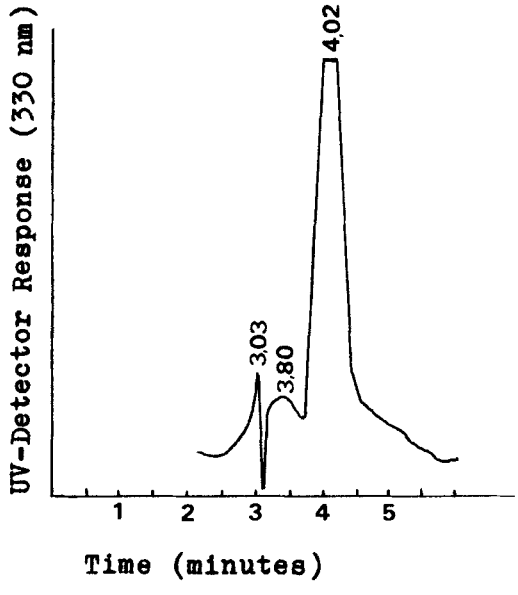


Figure 8: Chromatogram of an aqueous basic solution of III, after 24 hs.

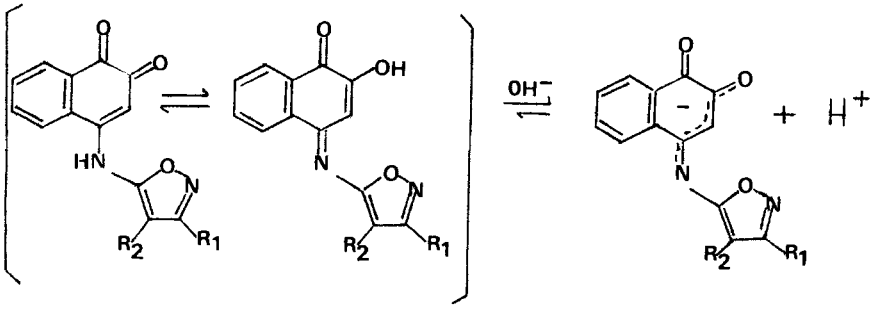
I and a second compound with a larger retention time than that of the enolic form (Figure 7).

Similar results were obtained when III was treated under similar conditions (Figure 8).

According to spectrophotometric data[7], we can assume an enol-enolate equilibrium given by the proton transfer reaction shown in Eq. 1.

These preliminary data on stability studies indicate that in ethanolic solutions the enol forms II and IV are more stable than the ceto forms I and III; whereas in aqueous solutions, at different pH values a ceto-enol equilibrium is established in all the cases.

It is concluded that this method is rapid, precise and exhibits adequate specificity for the analysis of compounds I-IV in samples from different origins as well as it is also useful as a HPLC stability-indicating assay because precursors and



Eq. 1

isomerization products can be determined simultaneously.

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