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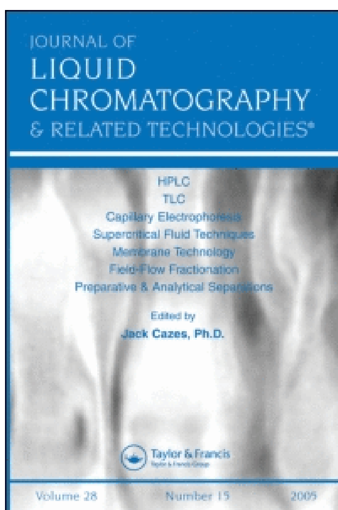

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HPLC SEPARATION OF TAUTOMERIC COMPOUNDS OF 4-AMINOISOXAZOLYL-1,2-NAPHTHOQUINONE. III.

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ABSTRACT

This paper reports the development of a simple, precise and specific HPLC procedure for the determination of two new methylated isoxazolylnaphthoquinones with an amine group in the 4-position of the isoxazole ring, which are examples of exocyclic tautomers.

The analytical procedure involved the use of the internal standardization method which was applied for the qualitative and quantitative determination of both compounds using a C-18 Lichrosorb column in an isocratic mode and sulfadiazine as internal standard.

INTRODUCTION

Previously we have reported[1,2] that the chromatographic (retention time, capacity factors and resolution) and the spectroscopic properties of several exocyclic tautomers derived from isoxazolyl-naphthoquinones proved to be useful for the separation and

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analysis by HPLC using silica gel column and ultraviolet detection. These structural characteristics allowed us to develop an accurate, precise, simple and rapid assay for compounds arising from 3- and 5-amino-methyl-isoxazoles. In this paper is investigated the applicability of this methodology for the analysis of two new methylated isoxazolylnaphthoquinones with the amine group in the 4-position of the isoxazole ring, which show structural stability differences compared with those previously described[3], using a C_{18} column, UV detector and methanol-water as the solvent system.

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Compounds:

N-(5-methyl-4-isoxazolyl)-4-amino-1,2-naphthoquinone (III), 2-Hydroxy-N-(5-methyl-4-isoxazolyl)-1,4-naphthoquinone-4-imine (IV) and 2-(5-methyl-4-isoxazolylamino)-N-(5-methyl-4-isoxazolyl)-1,4-naphthoquinone-4-imine (V) were obtained by the reaction between sodium-1,2-naphthoquinone-4-sulfonate (I) and 4-amino-5-methylisoxazole[4](II) in aqueous solutions.

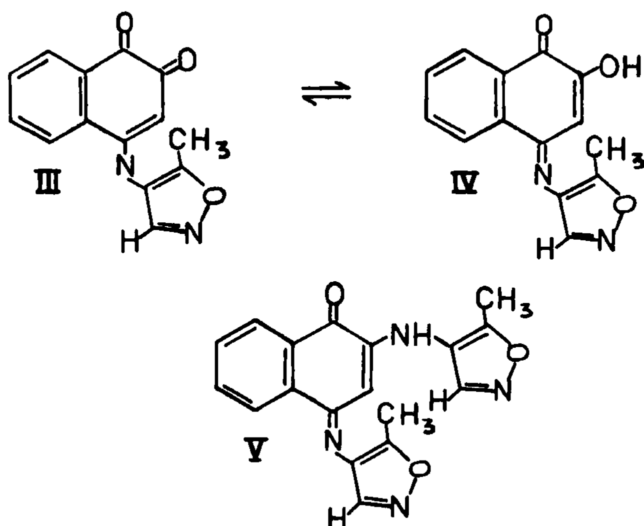

TABLE 1

Optimum Values of the HPLC Parameters

Column	RP-18 Lichrosorb (10 um) Hibar-Merck
Mobile Phase	Methanol-Water (50:50)
Volumen Injected	20 ul
Flow Rate	1.4 ml/min.
Detector	264 nm
Sensitivity	0.032 AUFS
Chart Speed	0.25 cm/min.

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These compounds offer the interesting possibility of examining the effect of structure on the distribution ratio of the keto-enol exocyclic tautomers III and IV in aqueous solution at different pH values and in solvents such as methanol, DMSO, and acetonitrile.

EXPERIMENTAL

Instruments

The chromatographic system (KONIK 500 G) consisted of an UVIS-200 variable wavelength detector, a Konik model SP-4290 Integrator and a Rheodyne Model 7125 injector with a 20 ul sample loop. The HPLC analyses were run isocratically using a reverse phase C-18 Lichrosorb column (250 x 4 mm, 10 um particle size, Hibar-Merck). The column was operated at room temperature. The optimum values of the established HPLC parameters are shown in Table 1.

Melting points were determined on a Buchi 510 melting points apparatus and were uncorrected. The IR spectra were recorded on a Nicolet 5-SXC FT-IR spectrophotometer from potassium bromide discs. ^1H-NMR spectra were obtained in $CDCl_3$ on a Bruker WP 80 SY at 80.13 MHz. Chemical shifts are reported in "delta" units. The mass spectra were recorded on a Finningan Model 3300 F-100. Quadrupole mass spectrometer data were collected and processed with an INCOS Data System using a Nova III Computer. A Cahn Electrobalance (Model G) was used to weigh the samples.

REAGENTS AND MATERIALS

All chemicals and reagents were of analytical grade. Methanol was treated with 2,4-dinitrophenylhydrazine according to the literature(8). Water for HPLC was purified with a Milli-Rho Milli-Q System (H_2O of 18 Mohm). Other solvents used were of analytical grade. The mobile phase was filtered in a Millipore system with pore size 0.45 um filters, degassed by vacuum and sonication before use. Citrate/HCl, phosphate and BO_3H_3/KCl/NaOH were used as the buffers for pH 4, 7 and 10 respectively.

N-(5-methyl-4-isoxazolyl)-4-amino-1,2-naphthoquinone (III).

This compound was synthesized by hydrolysis of V. Thus, the quinone-diimine V (0.001 mol, 0.403g) was dissolved in ethanol (50 ml) and then was heated under reflux in a water-bath (80ºC) for 10 min. An aqueous solution (25 ml) of 0.5N HCl was added dropwise while stirring and reflux was continued for 40 additional minutes. The cooled solution was extracted with dichloromethane (150 ml). The combined organic extracts were dried with anhydrous sodium sulfate, filtered and

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concentrated in vacuo yielding 0.259 g (84 %) of a red solid, which was recrystallized from benzene; mp. 209-209.5º. ^{1}H-NMR ($CDCl_3$): δ 2.411 (s,3H,CH_3); 5.757 (s,1H,H-3); 6.773 (br,1H,N-H); 7.678 (m,2H,H-7,H-6); 8.089 (m,2H,H-8,H-5); 8.284 (d,1H,H-3'). MS(25eV): m/z (%), 254(29); 239(9); 227(12); 185(26); 158(8); 43(100). IR (KBr): 1668 (C=O); 1615 (C=O); 3216 (N-H) cm^{-1}.

2-hydroxy-N-(5-methyl-4-isoxazolyl)-1,4-naphthoquinone-4-imine (IV).

To a solution of 0.004 mol (0.956 g) of sodium 1,2-naphthoquinone-4-sulfonate in phosphate buffer (pH=10.45, 50 ml), a solution of 0.003 mol (0.328 g) of 4-amino-5-methylisoxazole in 30 ml of water (pH=7) was added. The reaction mixture was stirred for 30 min at room temperature. The insoluble product formed was filtered and washed with water. Then it was recrystallized from ethanol; mp. 213-214º. ^{1}H-NMR ($CDCl_3$): δ 2.531 (s,3H,CH_3); 6.780 (s,1H,H-3); 7.220 (br,1H,O-H); 7.706 (m,2H,H-7,H-6); 8.208 (m,1H,H-8); 8.292 (d,1H, H-3'); 8.563 (m,1H,H-5). MS(70eV): m/z (%), 254(1); 227(2); 185(81); 158(24); 130(32); 43(100). IR (KBr): 3063 (O-H); 1691 (C=O); 1612 (C=N) cm^{-1}.

2-(5-methyl-4-isoxazolylamino)-N-(5-methyl-4-isoxazolyl)-1,4-naphthoquinone-4-imine (V).

This compound was prepared following a previously reported[6] procedure.

METHODS

Reference and Standard Solutions

A stock solution of the internal standard sulfadiazine (N^1-2-pyrimidylsulfanilamide) in methanol was

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prepared at a concentration of 10 ug/ml. This solution was stored at 5ºC and protected from light.

The stock solution of III and IV was prepared by dissolving an accurately weighed amount (0.5000 mg) of each of the compounds in 25 ml of the methanolic internal standard solution.

Calibration Curve

Serial dilutions of the stock solution (0.0200 mg/ml of each compound) were made with buffers at different pH values or solvents such as methanol, DMSO and acetonitrile to obtain five standard solutions ranging from 10 to 100 ug/ml.

Three injections of each standard solution were used to establish linearity and response ratios. The calibration curves were constructed by plotting a peak area ratio (analyte/internal standard) versus known analyte concentrations. Figures 1 and 2.

Preparation of Samples for Stability Studies

Stock solution of III and IV were prepared by quantitatively transferring 0.5000 mg of each compound to volumetric flasks of 25 ml capacity and adding the appropriate medium (buffers, methanol, DMSO and acetonitrile) until the volume was completed. An aliquot of 10 ml was introduced into a 10 ml volumetric flask. Then the flask was immersed in a constant temperature bath which was regulated by a thermostat (Haake F_3) with ±0.1ºC precision. At appropriate time intervals a 1.0 ml aliquot was transferred to a 5 ml volumetric flask and then diluted to the mark with internal standard stock solution and thoroughly mixed to obtain the standard working solution. A volume of 20 ul was injected in triplicate onto the column.

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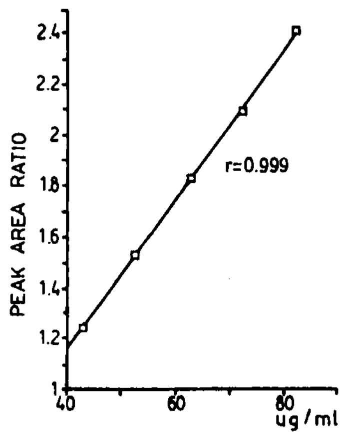

FIGURE 1: Compound III.
Solvent: Methanol.

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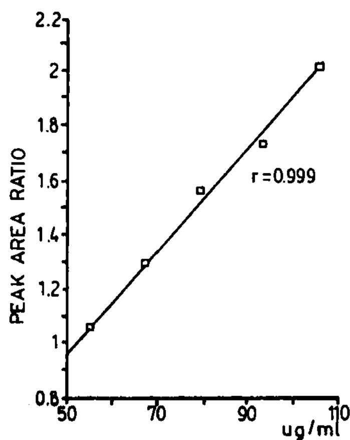

FIGURE 2: Compound IV.
Solvent: Methanol.

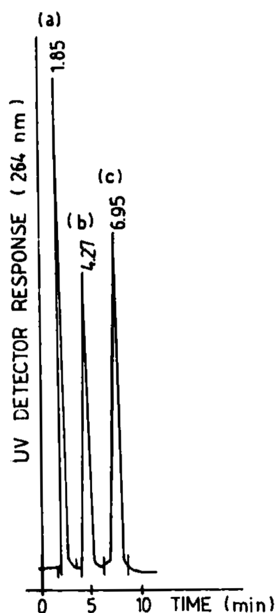

FIGURE 3: HPLC chromatograms of a standard mixture of compounds III and IV and the internal standard.

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RESULTS AND DISCUSSION

A chromatogram of a standard mixture of compounds III and IV as well as the internal standard are given in Figure 3.

The internal standard showed a peak (a) with retention time (R_t) of 1.85 minutes. Compounds IV and III showed peaks (b) and (c), having a retention time (R_t) of 4.27 and 6.95 minutes respectively. The total analysis time was ten minutes. From this chromatogram, it can be ascertained that the chromatographic working conditions found were excellent for the qualitative and quantitative determination of both compounds and for stability studies.

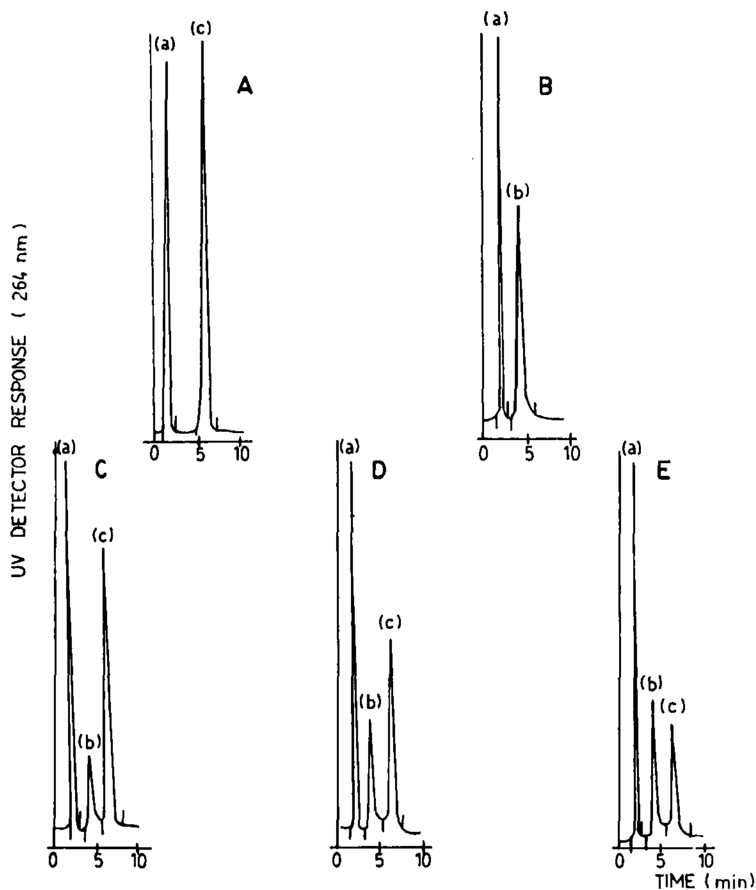

FIGURE 4: HPLC chromatograms of standard samples of isoxazolylnaphthoquinones. A: compound III; B: compound IV; C, D and E mixture of III + IV; 70:30, 50:50 and 30:70, respectively and the internal standard.

The selection of the internal standard was based on the fact that sulfadiazine (N^1-2-pyrimidylsulfanilamide), compared with at least 20 compounds, included the o-methylated fixed tautomers of IV, eluted close to III and IV with a well resolved peak.

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The mixture methanol-water (50:50) revealed to be the most successful among the five systems investigated, since it permitted the resolution of the tautomers in samples arising from the pure form as well as in combination. Figure 4.

The chromatographic resolution was calculated by equation 1:

$$R = \frac{(t_2 - t_1)}{1/2\ (w_2 + w_1)} = 1.86 \quad \text{eq. 1}$$

where t_2 and t_1 are retention times of the retained components III and IV respectively, measured at the peak maximum. The values for w_2 and w_1 are the peak widths in units of time measured at the base[7].

Linearity of the method with respect to concentration of compounds III and IV was checked. A good linearity was observed and a linear regression analysis of experimental data points showed a linear relationship with a good correlation coefficient.

The dependence of the chromatographic efficiency on flow rate was also investigated and the repeatability, accuracy, precision and sensitivity of the procedure were evaluated.

Table 2 shows the determined analytical method parameters.

From these data it can be deduced that the developed procedure is suitable for the analytical determination of both exocyclic tautomers III and IV in pure form and in combination.

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TABLE 2

Analytical Method Parameters

Parameters	Compounds	
	III	IV
Repeatability (M) n=5	2.6×10^{-7}	1.7×10^{-7}
(CV)	1.5	1.0
Precision[8] (CV) n=3	0.22	0.78
Sensitivity[9-10]	1.1	0.9
(MDQ-ng)[9] n=5	72	39
(MDC-M)[9] n=5	2.1×10^{-7}	1.8×10^{-7}

CV: Coefficient of Variation
MDQ: Minimum Detectable Quantity
MDC: Minimum Detectable Concentration

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Stability Studies

The stability indicating nature of the method was evaluated using solutions of III and IV (in water and different solvents) which were stored at 25ºC for 30 hours. No significant changes were observed in either of the tautomers during that period of time in methanol, DMSO and acetonitrile, suggesting that both compounds are stable in those conditions.

However, these results undergo variation in buffer solutions at different pH values. As long as the enolic tautomer IV remains stable at pH 4, 7 and 10 (25ºC) during the period of 30 hours, the keto form III un-

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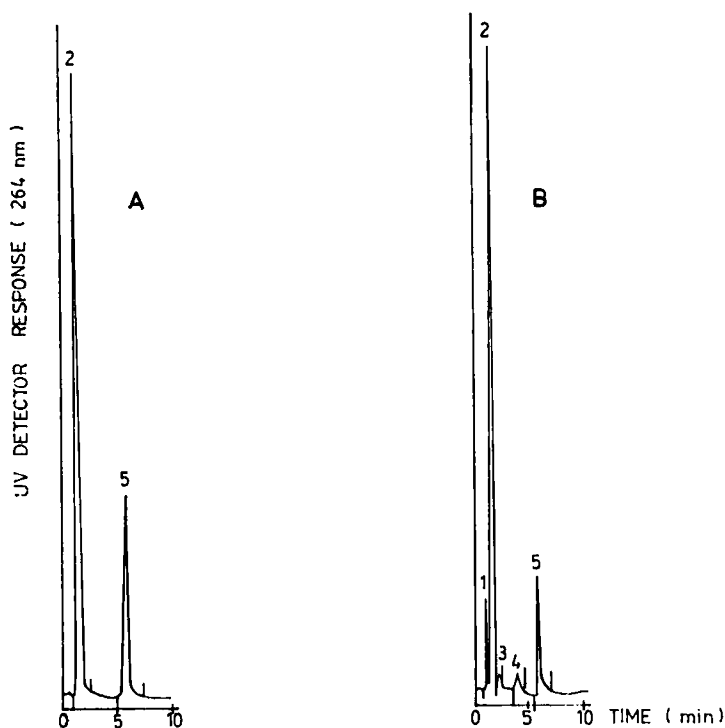

FIGURE 5: Chromatograms of compound III in buffer solution of pH=10. A: Initial condition t=0, 2 internal standard and 5 keto tautomer III. B: reaction time=6 hours, 1 and 3 hydrolysis products, 2 internal standard, 4 enolic tautomer IV and 5 keto tautomer III.

dergoes modifications leading to the corresponding isomer and also to hydrolysis products as it is shown in figure 5.

These results are consistent with those previously obtained[2], indicating that the rate of tautomeric change and other chemical modifications depend principally on the structure of the isoxazole unity as well as on the polarity of the solvents.

CONCLUSIONS

The procedure here described is sufficiently specific, sensitive, accurate and precise to analyse the exocyclic tautomers III and IV in the reaction medium, in pure forms, in combinations and it is also suitable as a stability indicating assay.

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REFERENCES

1. Fernández, A.; Bertorello, M.M. and Longhi, M.; "HPLC Separation of Tautomeric Compounds of 4-aminoisoxazolyl-1,2-Naphthoquinones". *J. of Liq. Chromatogr.*; 7 (11), 2203 1984.

2. Fernández, A. and Bertorello, M.M.; "HPLC Separation of Tautomeric Compounds of 4-aminoisoxazolyl-1,2-Naphthoquinones II." *J. of Liq. Chromatogr.*; 9 (13), 2843 1986.

3. Fernández, A.; Bertorello, M. M. y Manzo, R.; "Síntesis y Propiedades Espectroscópicas de 1,2-Naftoquinona-4-aminoisoxazoles" *Anales Asoc. Quím. Argentina*; 70, 49 1982.

4. Quilico, A.; Musante, C.; "Sopra i Nitro, Ammino e Diazoderivati Dell'isossazolo". *Gazz. Chim. Ital.*; 71, 327 1941.

5. Perrin, D.D.; Armanego, W. and Perrin D.R., "Purification of Laboratory Chemicals" Pergamon Press. Oxfor (England) 1982.

6. Ortiz, C. S.; Longhi, M. R.; Bertorello, M. M. y Briñón, M. C.; "Synthesis of Bisisoxazolylnaphthoquinones".
Org. Prep. and Proc. Int. 23, (2) 1991.

7. Snyder, L. R. and Kirkland, J. J.; "Introduction to Modern Liquid Chromatography", 2nd Ed., J. Willey, New York, 1979.

8. Kateman, G. y Pigpeers, F. W. "Quality Control in Analytical Chemistry", John Willey, New York, 1981.

9. Johnson y Stevenson "Basic Liquid Chromatography", Varian Associates, 1978.

10. Connors, K. A. "Reaction Mechanisms in Organic Analytical Chemistry", John Wiley, New York, 1973.

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