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Short Communication

Degradation profile and reversed-phase LC method development of the antiinflammatory drug, Ro 24-5913

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Keywords: *Leukotriene D_4 antagonist; degradation profile; reversed-phase liquid chromatography; photoisomerization; acid hydrolysis; pH sensitivity.*

Introduction

Leukotrienes are naturally occurring, biologically active, unsaturated lipids derived enzymatically from polyunsaturated acid precursors, such as arachidonic acid [1–3]. Recent publications have implicated LTD_4 as a mediator in asthmatic responses [4–7]. Ro 24-5913 ((*E*)-4-[[3-[2-(4-cyclobutyl-2-thiazolyl)-etheryl]phenyl]amino]-2-2-diethyl-4-oxobutanoic acid) has been selected as an experimental drug for treatment of asthma. This paper presents the development of a compound specific, reversed-phase liquid chromatography method and the degradation profile of Ro 24-5913. This analytical method will support the development of oral and metered dose inhaler formulations of Ro 24-5913 for use in both pre-clinical and clinical studies.

Experimental

Chemicals

All chemicals and solvents were of analytical and HPLC grade, unless otherwise indicated.

Instrumentation

The HPLC system consisted of a Waters Model 600E System Controller, Waters WISP Model 710B Autoinjector, and Applied Biosystems 783 Programmable Absorbance Detector operated at 240 nm wavelength or a Waters 990 Photodiode Array Detector. Ultraviolet spectra were determined with an HP 8452A Diode Array Spectrophotometer.

LC methods

Samples of 20 μl were injected under ambient conditions onto a reversed-phase Zorbax C_{18} (5 μm) (15 cm × 4.6 mm i.d.) column with a Zorbax reliance guard column (12.5 mm × 4 mm i.d.) containing C_{18} (5 μm) packing material. The flow rate was 1.0 ml min^{-1} using the following mobile phase systems.

System A. This system consisted of methanol–acetonitrile–buffer–2-propanol (55:20:20:5, v/v/v/v). The buffer was of tetrabutylammonium dihydrogen phosphate (5 mM) in 1% acetic acid adjusted with 6 N NH_4OH to pH 6.0.

System B. This system used the mobile phase of HPLC System A containing the buffer adjusted to pH 4.0.

System C. This system consisted of methanol–acetonitrile–buffer–2-propanol (50:28:20:2, v/v/v/v) with the same buffer used in HPLC System A, but adjusted to pH 5.0.

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System D. This system used the mobile phase of HPLC System C with the buffer adjusted to pH 5.5.

System E. This system used the mobile phase of HPLC System C with the buffer adjusted to pH 7.0.

System F. This system used the mobile phase of HPLC System D on Zorbax C_{18} (5 μm) (25 cm × 4.6 mm i.d.) column with a Zorbax reliance guard column (12.5 mm × 4 mm i.d.) containing the same packing material.

Semi-preparative LC system

The semi-preparative system consisted of a Waters Delta Prep3000 Preparative Chromatography System, Rheodyne 7010 Injector with 2.6 ml loop, Waters Delta Pak C18-100A, 15 μm, 30 cm × 7.8 mm i.d., semi-prep column with a Supelco Pelleguard C_{18} (2 cm) guard column, Waters Lambda-Max Model 480 LC Spectrophotometer, and LKB 2210 Recorder. The mobile phase consisted of methanol–acetonitrile–water–2-propanol (55:20:20:5, v/v/v/v). The chromatography was carried out on multiple 1-ml injections of the degraded sample solution using a flow rate of 4.0 ml min^{-1}. The fractions corresponding to principal degradation product from the photolytic degradation of the methanolic solution of **1** were collected and concentrated with a rotary evaporator.

Photolytic degradation — solid state

Approximately 30 mg of **1** was placed in a quartz vial and exposed to short wave (254 nm) high intensity ultraviolet light for up to 24 h. After 4 and 24 h, a fraction of approximately 12 mg was withdrawn and weighed accurately and dissolved in 25 ml of methanol.

Photolytic degradation — solution state

A 0.5 mg ml^{-1} solution of **1** was prepared by dissolving 50 mg in 100 ml of methanol (Solution A). A 15-ml aliquot of Solution A was transferred into a 25-ml clear borosilicate flask and placed in a Cooper–Lackman fluorescent light chamber (light intensity: 400 ft candles) for up to 24 h. After 4 and 24 h, an aliquot of 5.0 ml was withdrawn and diluted to 10 ml with methanol.

Hydrolytic degradation — acid hydrolysis

A 10-ml aliquot of Solution A was placed in a 100-ml flat bottomed amber flask equipped with a reflux condenser; 10 ml of 0.2 N HCl was added and the contents refluxed on a steam bath for 1 h. The reaction mixture was cooled and diluted to 50 ml with methanol.

Hydrolytic degradation — base hydrolysis

A 10-ml aliquot of Solution A was placed along with 10 ml of 0.2 N NaOH in a 100-ml flat bottomed amber flask equipped with a reflux condenser. The contents were refluxed on a steam bath for 1 h. The reaction mixture was cooled and diluted to 50 ml with methanol.

LC response factors

The response factors at 240 nm were calculated for **1** and for each of the degradation products by dividing the respective area response by the individual concentration in μg ml^{-1}. Relative response factors (RRF) were calculated by dividing the response factor of **1** by the response factor of the degradation product. Per cent purity by area normalization and relative response factors (RRF) of compounds **1–5** are given in Table 1.

Results and Discussion

The chemical structures of **1** and its degradation products (**2–5**) are given in Fig. 1. Compound **3** is an immediate precursor and is a potential impurity in the synthesis of **1**. An initial LC method was developed for the separation of **1** and **3** using LC System B.

Table 1
Chromatographic data of Ro 24-5913 and degradation products/impurities using HPLC System F

Component	Retention time, t_R	Relative retention time	Relative response factor at 240 nm	Purity by area normalization
1	12.0	1.0	1.0	98.9
2	7.9	0.6	1.0	98.5
3	10.4	0.9	1.2	87.1
4	22.1	1.8	2.1	98.9
5	23.4	1.9	1.4	99.6

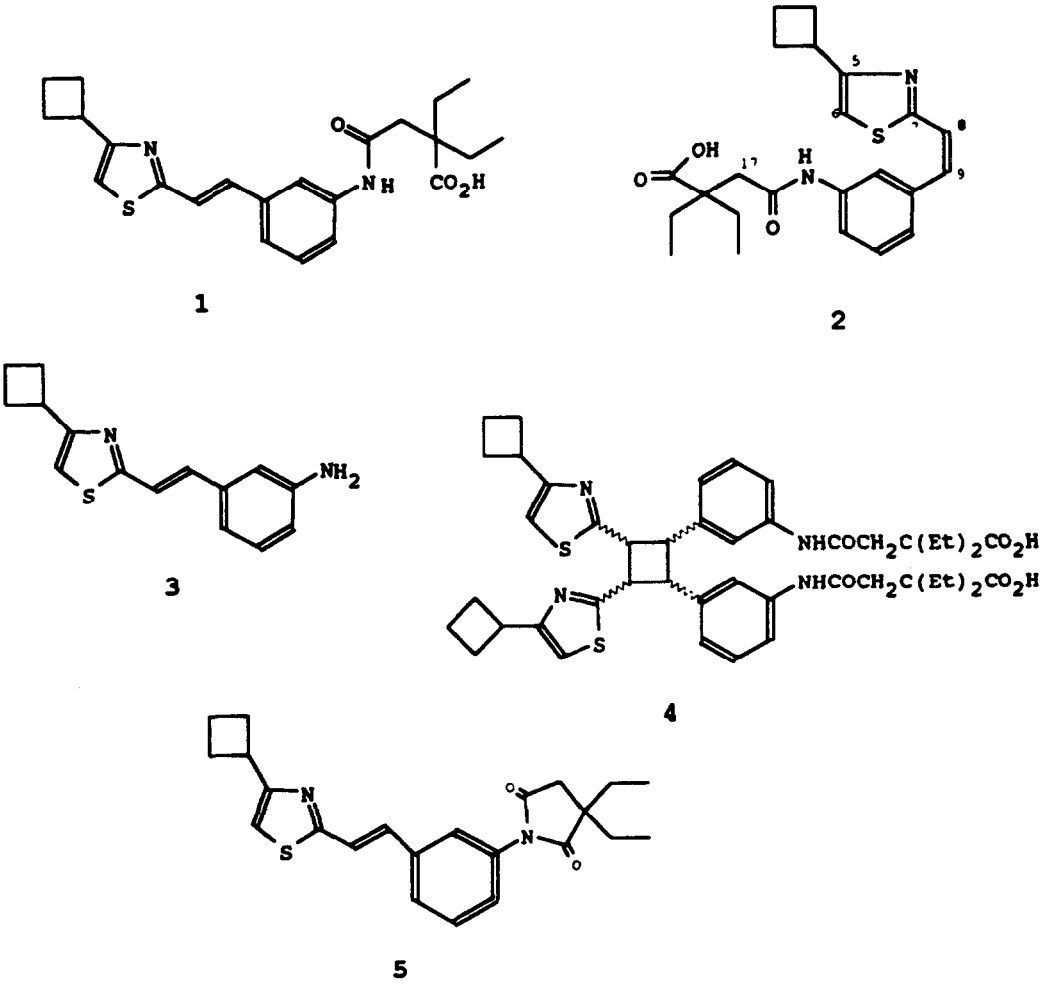


Figure 1
Chemical structures of (**1**) Ro 24-5913, (**2**) Ro 24-9921, (**3**) Ro 24-6496, (**4**) Ro 24-9971 and (**5**) Ro 24-6593.

However, the stressed solutions contained additional peaks with retention times similar to **1** which made it necessary to develop a system capable of resolving these degradation products. Table 2 presents the experimental conditions, the amounts of degradation products and impurities formed, and the mass balance (%).

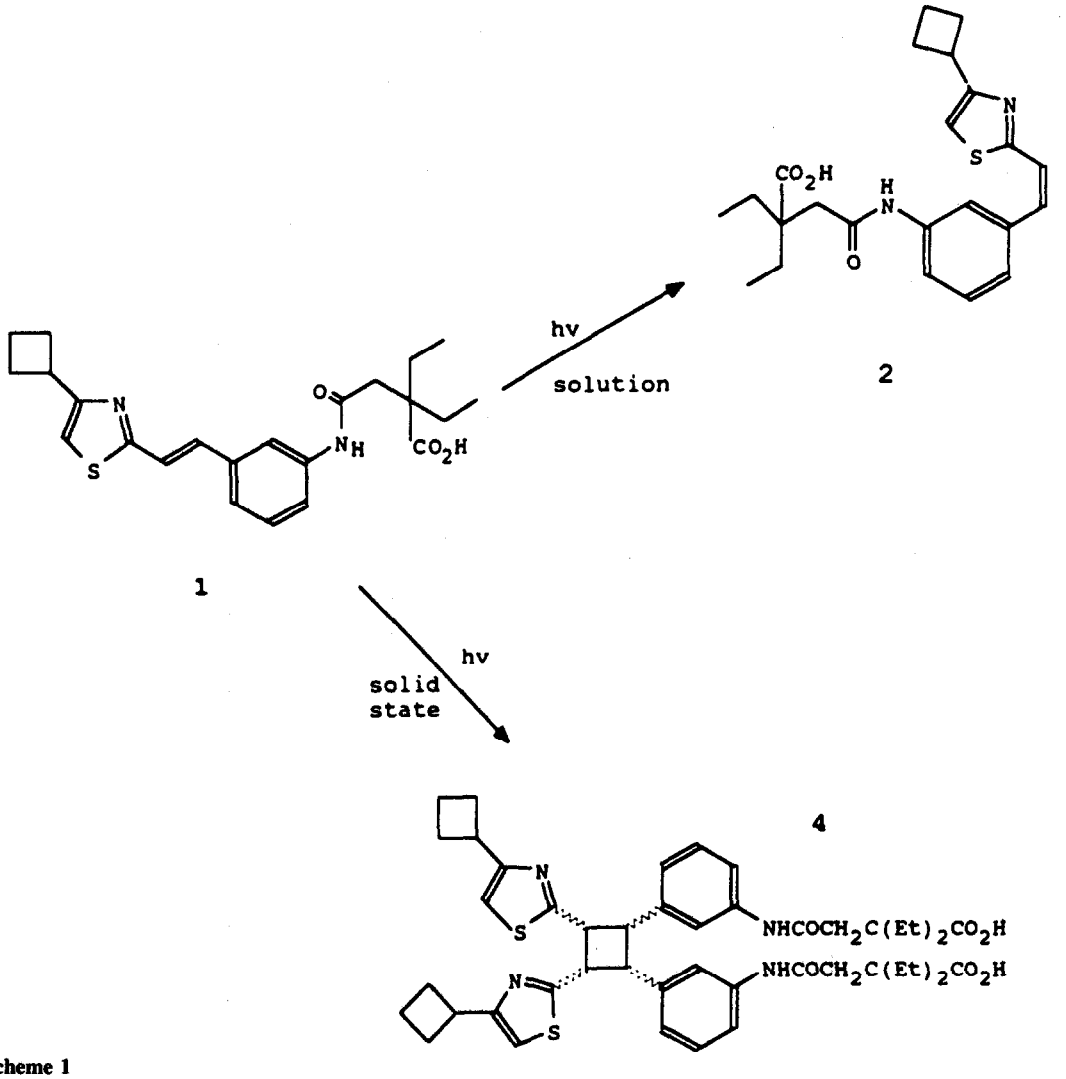
Exposure of a methanolic solution (Solution A) of **1** to fluorescent light for 24 h formed an additional peak at a retention time of 2.9 min using System A as shown in Fig. 2(A). This degradation product was formed in 65% yield by area per cent and was characterized as the *cis* isomer (**2**) by ^{1}H-NMR (DMSO-d6): δ 0.79 (t, 6H, CH_3), 1.64 (q, 4H, CH_2), 1.90 (m, 2H, ring CH_2), 2.19 (m, 4H, ring CH_2), 2.57 (s, 2H, $COCH_2$), 3.53 (m, 1H, ring CH), 6.83 (d, 1H, J = 12 Hz, *cis* CH), 6.88 (d, 1H, J = 12 Hz, *cis* CH), 7.17 (d, 1H, ArH), 7.31 (t, 1H, ArH), 7.55 (d, 1H, ArH), 7.66 (s, 1H, ArH), 9.91 (s, 1H, NH); ^{13}C-NMR ($CDCl_3$/DMSO): δ 8.7 (C-20, C-22), 18.5 (C-3), 28.4 (C-2, C-4), 28.9 (C-19, C-21), 36.5 (C-1), 41.5 (C-17), 49.1 (C-18), 112.8 (C-8), 119.6 (C-5), 119.8 (C-9), 123.9 (C-13), 124.6 (C-11), 129.1 (C-15), 134 (C-14), 136.8 (C-7), 139.1 (C-10), 160.6 (C-12), 163.5 (C-16), 170.2 (C-6), and 178.9 (C-23); FT-IR (KBr) 780 (—CH=CH—) cm^{-1}; MS: *m/z* 412 (M^+); UV (λ_{max}, MeOH): 208, 244, and 306 nm. When **1** in solid state was exposed to high intensity ultraviolet light for 24 h, a new degradation product was formed at a retention time of 4.8 min using System A as shown in Fig. 2(B), tentatively identified as **4**. The photolytic degradation pathway of **1** is shown in Scheme 1.

The hydrolytic degradation pathway of **1** is shown in Scheme 2. Refluxing **1** in 0.1 N HCl–MeOH (1:1) for 1 h on a steam bath formed

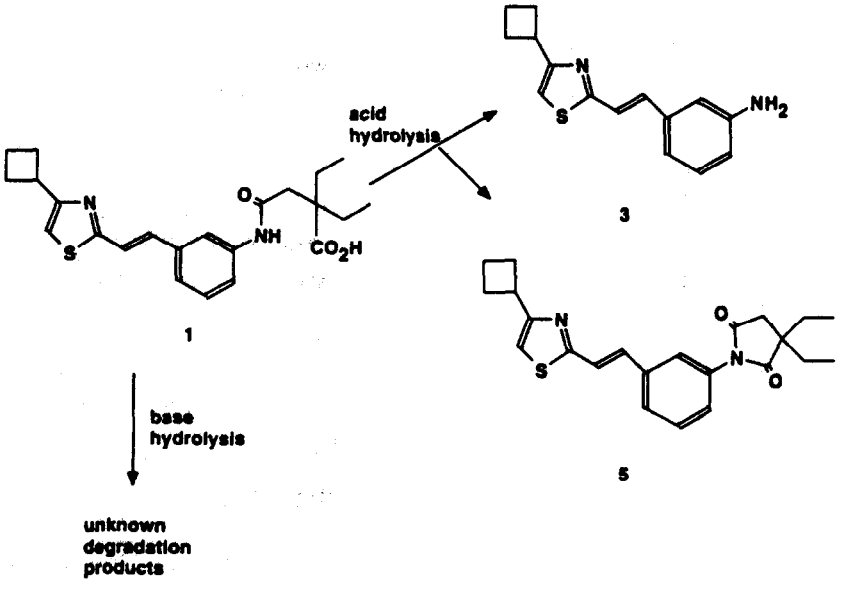
Table 2
Degradation of Ro 24-5913

Degradation conditions	Degradation products found: Compound (RRT) (m moles)					Degradation products found: % Area normalization					Mass balance (%)
	2 (0.72)	**1** (1.00)	**3** (1.09)	**4** (1.69)	**5** (1.74)	**2** (0.72)	**1** (1.00)	3 (1.09)	**4** (1.69)	**5** (1.74)	
Methanolic solution exposed to fluorescent light[2] for 4 h	0.044	0.442	—	—	—	2.1	90.9	—	—	—	99.6[5]
Methanolic solution exposed to fluorescent light for 24 h	0.319	0.167	—	—	—	65.2	34.1	—	—	—	100[5]
Solid exposed to ultraviolet light for 4 h[3]	0.001	0.482	—	0.021	—	0.2	97.5	—	2.2	—	103[5]
Solid exposed to ultraviolet light for 24 h	0.001	0.366	—	0.136	—	0.2	83.5	—	16.2	—	103[5]
Methanolic–0.1 N HCl solution refluxed for 1 h[4]	—	0.011	0.462	—	0.08	0.1	3.7	79.0	—	16.5	114[4]
Methanolic–0.1 N NaOH solution refluxed for 1 h	0.019	0.464	—	—	—	3.9	96.0	—	—	—	99.6[4]

[1]Known compounds were calculated using experimentally determined relative response factors (RRF) to Ro 24-5913. For unknowns, the RRF was taken to be unity and the molecular weight was approximated to be equivalent to Ro 24-5913.
[2]Cooper–Lackman Light Chamber (400 ft Candles).
[3]0.3% unknown at RRT of 0.57 was present.
[4]0.1% unknown at RRT of 1.41 and 0.5% of unknown at RRT of 2.32 were present.
[5]The molarity of the control was 0.488 mM. HPLC System B was used.
[6]The molarity of the control was 0.485 mM. HPLC System A was used.



Scheme 1
Photolytic degradation of Ro 24-5913.



Scheme 2
Hydrolytic degradation pathway for Ro 24-5913.

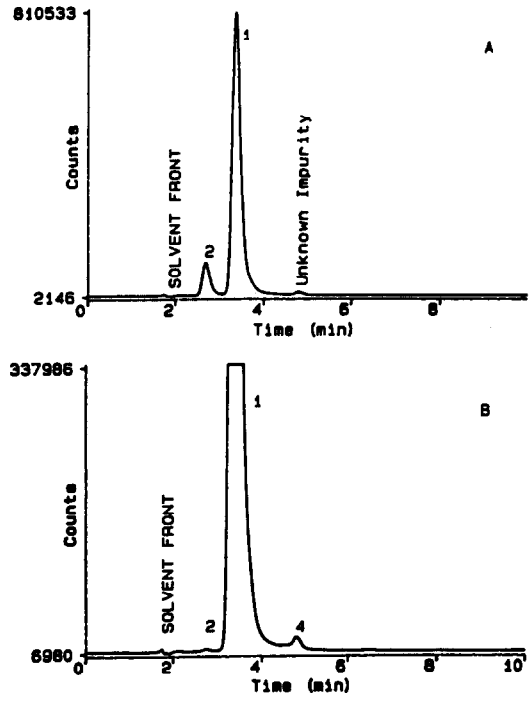


Figure 2
Chromatograms for photolytic degradation of Ro 24-5913 using HPLC System A. (A) Methanolic solution exposed to fluorescent light for 4 h, (B) solid exposed to high intensity ultraviolet light for 24 h.

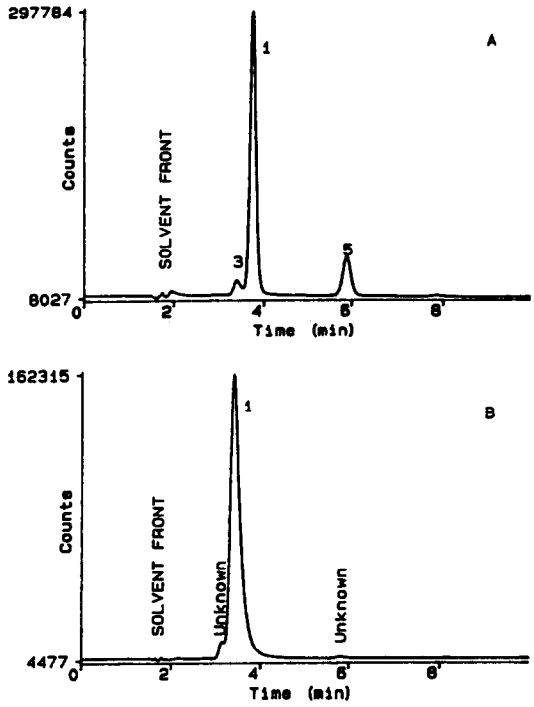


Figure 3
Chromatograms of Ro 24-5913 hydrolysis products using HPLC System A. (A) Methanolic–0.1 N HCl solution refluxed for 1 h, (B) methanolic–0.1 N NaOH solution refluxed for 1 h.

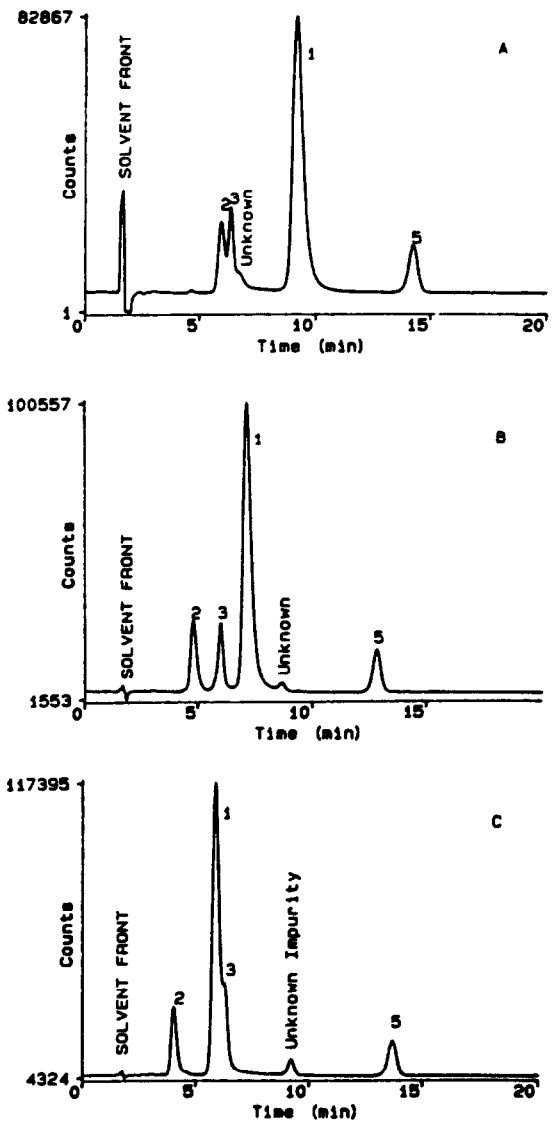


Figure 4
Chromatograms showing the effect of mobile phase pH on the separation of Ro 24-5913 and degradation products. (A) pH 5, (B) pH 5.5, (C) pH 7.

79% of **3** and 16.5% of **5** as the major degradation products along with minor peaks attributed to **2** and **1** [Fig. 3(A)]. Acid catalysed ring closure of the succinamide moiety was favoured which yielded **5** as the predominant product. Compound **1** was far less sensitive toward base hydrolysis as compared to acid hydrolysis. However, small amounts of unknown products were formed when **1** was refluxed in 0.1 N NaOH–methanol solution as shown in Fig. 3(B).

During HPLC method development, **1** and **2** were found to be highly sensitive to changes in mobile phase pH as shown in Figs 4 and 5. The

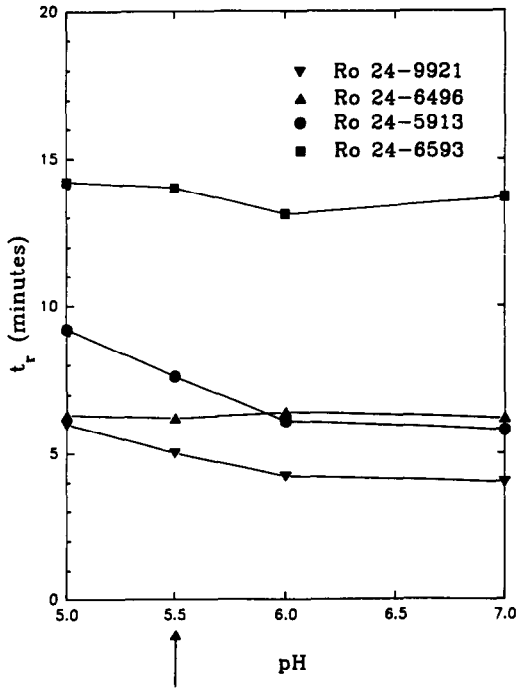


Figure 5
Effect of buffer pH on peak retention times.

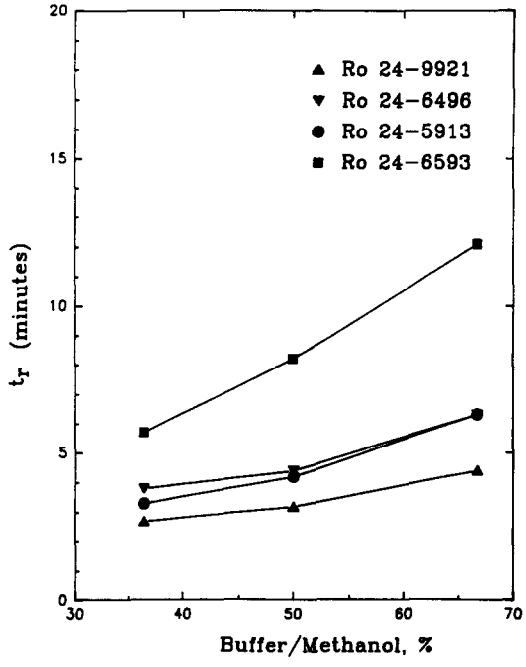


Figure 6
Effect of methanol concentration at fixed buffer pH 5.5 on peak retention times.

effect of methanol concentration at a fixed buffer pH of 5.5 on peak retention times is shown in Fig. 6. Using HPLC Method D, **4** appeared at the front of **5**. A longer C_{18} column (25 cm) at buffer pH 5.5 using HPLC System F provided a satisfactory separation of

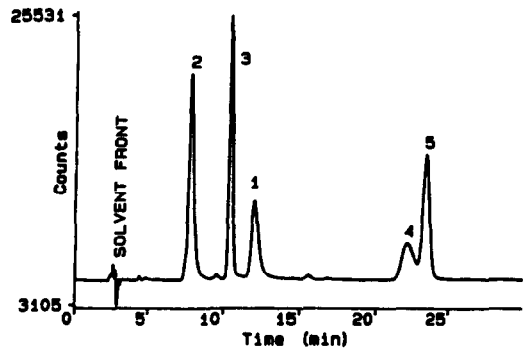


Figure 7
Chromatogram of a spiked mixture of Ro 24-5913 and degradation products using HPLC System F.

Ro 24-5913, the degradation products and unknowns as shown in Fig. 7.

Conclusions

A reversed-phase LC method was developed for the evaluation of Ro 24-5913. This compound undergoes primarily photolytic and hydrolytic degradation. Mass balance was obtained for the degradation pathways evaluated. The major light-induced degradation product of **1** in solution was isolated and structurally identified as the *cis* isomer (**2**).

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