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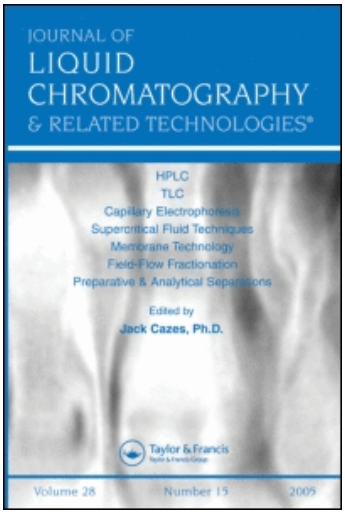

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Determination of 4-epi-Meclocycline, a Tetracycline Analog, in Cream Formulation by HPLC and HPTLC

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DETERMINATION OF 4-EPI-MECLOCYCLINE, A TETRACYCLINE ANALOG, IN CREAM FORMULATION BY HPLC AND HPTLC

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ABSTRACT

An unidentified peak has been observed in the chromatograms of thermally stressed meclocycline sulfosalicylate creams by high performance liquid chromatography (HPLC). The unknown has been isolated by preparative HPLC and by high-performance thin layer chromatographic (HPTLC) methods and identified to be a reversible isomer, the 4-epimer, of meclocycline, a tetracycline analog. The identification has been accomplished by matching the ultraviolet, mass, and circular dichroism spectra of the unknown with those of the synthetic epimer. The conversion of meclocycline to the 4-epimer has been achieved in glacial acetic acid and the product purified by HPTLC. The 4-epimer has been found to be easily converted to the original meclocycline in hot ethanol. The data indicate the epimerization not to be the favored reaction, but rather an equilibrium state which favors the intact meclocycline.

INTRODUCTION

The current marketed cream formulation of the antibiotic, meclocycline sulfosalicylate (1), (I, Fig. 1), is recommended for the topical treatment of acne. Meclocycline base is a 7-chloro-6-methylene-5-hydroxy-derivative of tetracycline. Although no degradation products of meclocycline have been previously reported, a small unknown peak has been observed in

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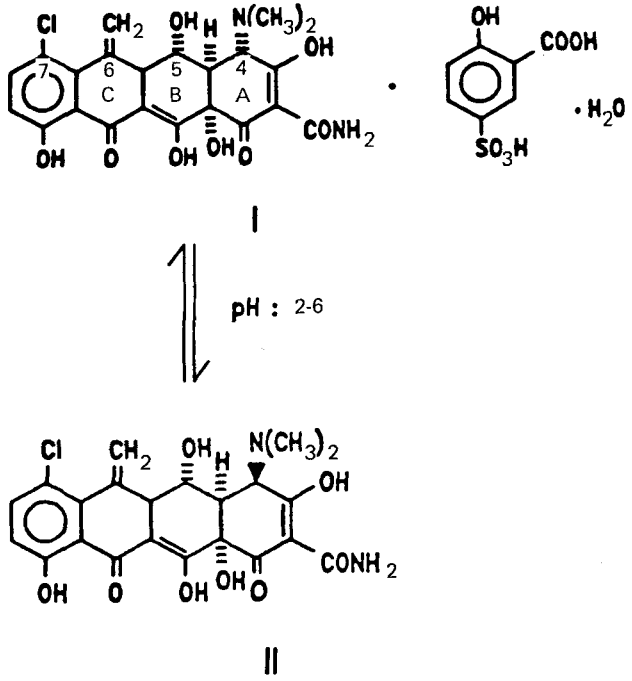

Figure 1: Structures of Meclocycline Sulfosalicylate (I) and its 4-epimer (II).

the chromatograms of some temperature-stressed cream samples by a recently developed high-performance liquid chromatography (HPLC) assay method(2).

In general, the major degradative pathways for tetracyclines have been reported to be epimerization, dehydration, hydrolysis and oxidation(3,4). However, only epimerization and dehydration are most commonly encountered, especially under the relatively mild environmental conditions to which pharmaceutical preparations are normally subjected. The absence of the C-6 hydroxyl group in meclocycline precludes the dehydration and concomitant aromatization to the anhydro-derivative which occurs so readily in most tetracycline analogs. This would suggest epimerization as the only significant degradative route for meclocycline.

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Initially, the separation of tetracyclines and their epimers was accomplished by partition chromatography and differential crystallization(5). Several thin layer chromatographic (TLC) methods of separation have also been applied to tetracyclines and their degradation products(6-11). Most of the above techniques were either not applicable to meclocycline and its epimer or were too cumbersome to give reliable and reproducible results. One exception was the TLC system employing edetic acid-treated silica gel plates and a developing solvent consisting of methyl ethyl ketone saturated with McIlvaine's buffer(9). Several HPLC systems developed for tetracyclines and their breakdown products have been extensively reviewed (12). However, it was found that the best HPLC system for meclocycline was based on a reversed-phase Vydac column as reported for a few tetracycline analogs(13). Using this column and a mobile phase of tetrahydrofuran in ammonium edetate(2), an occasional unknown peak was observed in chromatograms during stability analyses of aged meclocycline sulfosalicylate creams.

This paper describes how the suspected epimer was first synthesized and purified by high-performance TLC (HPTLC) from raw material, and then isolated from temperature-stressed cream formulation by HPLC and HPTLC and identified by ultraviolet, mass, nmr and circular dichroism spectroscopy.

EXPERIMENTAL

Materials

Meclocycline sulfosalicylate (I) and methacycline, 6-methylene-5-hydroxytetracycline (Pfizer, Inc., New York, NY) were used in all experiments as received. For the cream samples, a 1% (w/w) formulation of meclocycline sulfosalicylate cream(1) was used. Tetrahydrofuran (Burdick and Jackson Labs Inc., Muskegon, MI) was HPLC grade and (ethylenedinitrilo)-tetraacetic acid (edetic acid) (J.T. Baker Chemical Co.,

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Phillipsburg, NJ) and all the other chemicals and solvents were analytical reagent grade.

High Performance Liquid Chromatography

Both analytical and preparative HPLC work were performed on a Waters Associates M224 liquid chromatograph equipped with M6000A pumping system and M440 UV detector operated at 340 nm (Waters Associates, Milford, MA). A syringe-loaded injection valve with either a 10 µl loop (Valco Valve, Valco Instrument Co., Houston, TX) or alternatively a 2 ml loop (Waters Associates Model U6K Universal Injector, Milford, MA) was used. The columns were packed with octadecylsilane material bonded to microparticulate silica gel (10 µm), Vydac 201 TP Reversed Phase (25 cm x 3.2 mm I.D.) for analytical work and Vydac 201 Reversed Phase (25 cm x 10 mm I.D.) for preparative work (The Separations Group, Hesperia, CA). The 0.001 M ammonium edetate needed for the mobile phase was prepared by mixing 0.6 g of edetic acid with 2 ml of methanol and dissolving in 15 ml of concentrated ammonia. Approximately 1800 ml of water were added, and the pH was adjusted to precisely 6.6 with glacial acetic acid. Water was added to make a final volume of two liters. The solution was filtered through a fluoropore membrane filter (0.5 µm) and mixed with tetrahydrofuran in a ratio of 85:15 (v/v) for analytical and 87:13 (v/v) for preparative work as the mobile phase. The flow rates were 0.8 ml/min for analytical and 3.5 ml/min for preparative columns. Columns were periodically purged with 95% ethyl alcohol (Form III) (Sargent-Welch Sci., Co., Skokie, IL) for maximum performance. A 10-mV strip chart recorder was employed at a chart speed of 0.5 cm/min. Samples were dissolved in methanol and diluted with mobile phase before injection.

Thin Layer Chromatography

The TLC system consisted of high-performance silica gel plates, LHP-K, 10 x 10 cm (0.2 mm) with a preadsorbent layer

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for sample application (Whatman Inc., Clifton, NJ). The plates were presoaked in 0.1 M disodium edetate solution for 1 hr and activated by drying with a warm air-dryer for a minimum of 1 hr. The preadsorbent area was left above the soaking solution. The developing solvent was prepared by saturating methyl ethyl ketone (99%) with McIlvaine's buffer - a dibasic sodium phosphate (0.2 M) and citric acid (0.1 M) buffer - adjusted to pH 4.7. All the visualizations were done by direct observation under long wavelength UV light.

Spectroscopic Instrumentation

NMR spectra were recorded on a Varian FT 80A 80 MHz instrument in the Fourier transform mode in d_7-dimethylformamide using tetramethylsilane as internal reference at ambient temperature. The chemical shift values were recorded in ppm and were calculated from cps data. UV spectra were recorded with a double beam Perkin Elmer 554 Spectrophotometer in 1-cm quartz cells at ambient temperature using the HPLC mobile phase as solvent for sample preparation and in the reference cell. Mass spectra were obtained by solid probe technique on a Finnegan 1015-D mass spectrometer with a 6100 Data System. The spectrum of each compound was obtained at an ionizing energy of 70eV. Circular dichroism spectra of the samples were determined in a 1-cm path length cell at ambient temperature using a Cary-60 Spectropolarimeter equipped with a Cary-6001 circular dichroism accessory.

Preparation of Mixture of I and 4-Epi-I (II)

A solution of I (200 mg, 0.28 mmoles) in 100 ml of glacial acetic acid was heated on a steam bath for 2 hr. The acid was evaporated using a stream of nitrogen. The residue was reconstituted in 10 ml of methanol and kept refrigerated overnight. This procedure caused some I to precipitate, and a filtrate enriched with the product (II) was achieved. The product content of the filtrate was 30-40% of the total

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mixture, based on area % quantitation by HPLC. ^{1}H NMR (DMF-d_7), δ2.56 (S, 6H, $(CH_3)_2N$ for I), 2.69 (S, 6H, $(CH_3)_2N$ for II), 4.03 (S, 1H, H-4 for I), 4.21 (S, 1H, H-4 for II), 4.70-4.85 (m, 4H, methylene H for I and II), 5.60-5.68 (m, 4H, aromatic H for I and II). The mixture was used in the next step of separation by HPLC and HPTLC.

Isolation of 4-Epi-I(II) from Mixture of I and II by HPLC

An aliquot of the filtrate (mixture of I and II in methanol) was mixed with mobile phase (1:1, v/v) and chromatographed on a preparative reversed-phase column using the mobile phase of tetrahydrofuran in edetate buffer at pH 6.6 (87:13 v/v). The peaks corresponding to I and II were collected and reanalyzed on an analytical column for isomer purity. Both collections were shown to be over 99% pure based on area % quantitation by HPLC. The UV spectra of the collected peaks in the mobile phase (2.46×10^{-5} M for I and 4.78×10^{-5} M for II) showed λmax at 238 nm (E_{238} 26000), 280 nm (E_{280} 19500) and 350 nm (E_{350} 14200) for I and 235 nm (E_{235} 12600), 270 nm, shoulder (E_{270} 8200) and 370 nm (E_{370} 6700) for II. The HPLC collections were freeze-dried and the residues were extracted with methanol. However, the methanol extract showed excessive amount of ammonium edetate which interfered in the mass spectral and nmr determinations of the product. Ethanol extracted less of ammonium edetate but some heating was required in the dissolution, and this resulted in almost complete reversal of the product to I.

Isolation of 4-Epi-I(II) from Mixture of I and II by HPTLC

An aliquot of the filtrate (mixture of I and II in methanol) was streaked on the preadsorbent layer of HPTLC plate (pre-treated with disodium edetate). The plate was developed in methyl ethyl ketone saturated with McIlvaine's buffer at pH 4.7. Two readily discernible bands for I and II were developed with R_f values of 0.24 and 0.09, respectively. The small amount

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of methacycline impurity (R_f ~ 0.19) coeluted with I. Visualization was by long wavelength UV light. The silica gel bands were scraped off the plates, mixed with methanol, centrifuged and the extracts evaporated to dryness. Although the edetate salt was present again, this was not a major problem, since disodium edetate is not very soluble in methanol (ammonium edetate is soluble in methanol). However, methanol extracted from the silica gel some extra material which interfered with both mass spectral and nmr determinations. When the developing solvent was used instead of methanol for the extraction, the yellow crystalline product from band II was shown to be over 90% pure II by HPLC area % integration. The rest of the material was I, either converted from II during preparation or from tailing of I on the plate. Band I was shown to be over 99% pure I. MS for both I and II: m/z 476 (M^+, isotopic cluster of 476:478 in ratio of approximately 3:1). CD for I: λ^t 260 nm ($[\theta]^t$ -3.8 x 10^4), λ^pmax 290 nm ($[\theta]^p$ 2.2 x 10^4), λ^t 330 nm ($[\theta]^t$ -1.0 x 10^4). CD for II: λ^p 245 nm ($[\theta]^p$ 0.4 x 10^4), λ^pmax 280 nm ($[\theta]^p$ 0.8 x 10^4), λ^tmax 305 nm ($[\theta]^t$ -1.1 x 10^4).

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Isolation of 4-Epi-I(II) from Aged Pharmaceutical Preparations

In order to isolate the unknown decomposition product from aged cream preparations of I, a significantly stressed formulation if I (about 4% epimerization) was selected and extracted with methanol. The yellow methanol extract was streaked directly onto the preadsorbent area of the high-performance TLC plate (pretreated with disodium edetate solution). Two sharp bands were observed using methyl ethyl ketone saturated with McIlvaine's buffer at pH 4.7 for development (R_f ~0.20 for I and R_f ~0.09 for II). The small amount of methacycline impurity (R_f ~0.19) coeluted with I. The bands corresponding to compounds I and II were scraped off the plate, extracted

with developing solvent, centrifuged and the extracts evaporated to dryness. By using HPLC area % integration, the band of I was shown to be over 99% pure and the band of II was 73-88% pure. The rest of the band II was again I, either converted from II during preparation or from residual tailing of I on the same plate. The MS and CD spectra of band II exhibited peaks similar to those observed in the MS and CD spectra of synthetic II, isolated from mixture of I and II.

RESULTS AND DISCUSSION

The amount of degradation of I in cream formulations, shown by HPLC, was quite minimal as might be expected for solid suspensions. Stability data for representative aged samples showed less than 1% of the product in HPLC chromatograms even after a storage of a year and a half at room temperature. Typical chromatograms for a standard I and a cream sample of I with the suspected 4-epimer (II) present are shown in Fig. 2. The system gave a baseline separation for I and II, peaks B and C, respectively, and for methacycline, peak A, an impurity always present in raw material but never present at more than 3% of the total weight. The retention times were about 5 minutes for methacycline, 7 minutes for II and 8.5 minutes for I. The retention times for I and impurities could be easily shifted by varying the relative amount of tetrahydrofuran in the mobile phase. It was preferred, however, to keep the retention time of I in the range of 5.5 and 8.5 minutes.

The 4-epimer of I, an inactive and nontoxic drug substance (II), was being considered the most likely candidate for the unknown peak because of the following facts. The pH of the cream preparations of I is about 3, which is favorable, based on the chemistry of tetracyclines, to the formation of 4-epimer(5). Also, the 4-epimers of tetracyclines have been shown to elute before the parent compounds in reverse phase

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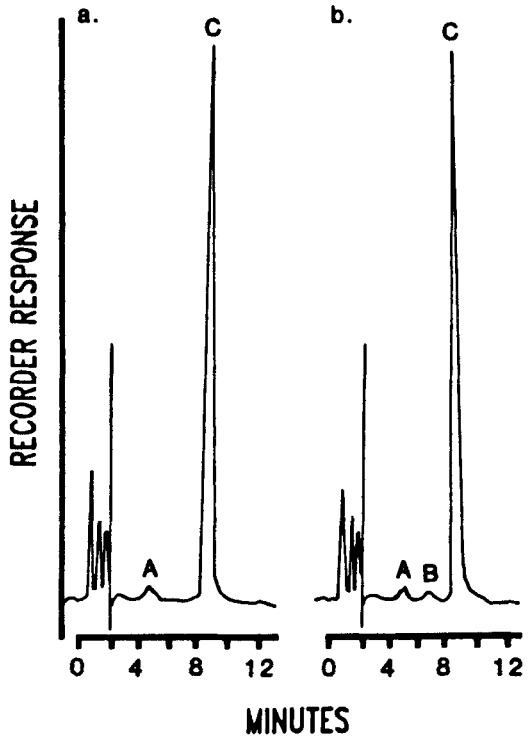

Figure 2: Chromatograms of meclocycline sulfosalicylate (I) standard (a) and a cream sample of I, aged for 30 days at 50° (b). Peak A: methacycline, Peak B: 4-epi-I, Peak C: I. Conditions: mobile phase, 0.001 M ammonium edetate, pH 6.6-tetrahydrofuran (85:15, v/v), pH adjusted with glacial acetic acid; column, Vydac 201 TP Reversed Phase, 25 cm x 3.2 mm I.D.; flow rate, 0.8 ml/min; detector, UV at 340 nm.

HPLC systems (13). However, no such epimer of I was available for testing, and no previous methods for its preparation were known either. Therefore, a synthetic scheme for preparation of 4-epi-I had to be devised. The synthesis was based on the fact that saturated solutions of tetracyclines in glacial acetic acid have been shown to equilibrate within 24 hours(5). The most favorable conditions for the C-4 epimerization of I were

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found to be a saturated solution of I in hot glacial acetic acid heated on a steam bath for 2 hr. After this time, equilibrium was reached with the conversion of 20-30% of I. Higher temperatures and longer heating times did not produce more of the suspected epimer. However, a filtrate containing 30-40% of II could be achieved by partial precipitation of I. A typical chromatogram of such an equilibrium mixture is shown in Fig. 3a. A comparison of the NMR spectra in d_7-dimethylformamide for I and the equilibrium mixture showed the mixture to have new peaks with typical chemical shifts to lower field of the C-4 proton (from 4.03 ppm to 4.21 ppm) and the dimethyl protons of the C-4 amino group (from 2.56 ppm to 2.69 ppm) relative to I, indicative of an epimer. Similar trends in the chemical shifts have been found in NMR spectra of tetracycline and its 4-epimer(14,15).

Since an HPLC method effecting separation and quantitation of I and its breakdown product existed, and the respective preparative column was available, preparative HPLC separation of the product from I was attempted. This method gave at least 99% pure product relative to I, based on area % quantitation by HPLC (Fig. 3b), but further clean-up from the mobile phase proved difficult. One difficulty encountered was the fact that depending on reaction conditions, the product could be converted back to I. When the purified product was heated in ethanol on steam bath for half an hour, almost complete reversal (~98%) back to I was observed by HPLC. However, good UV spectra of both the product and I were recorded directly on the HPLC collections (Fig. 4). They were very similar and showed typical absorbance patterns reported for 4-epimer and the parent compound of other tetracyclines(5). The absence of absorbance in the 400-500 nm region was strong evidence, in addition to the structural features, that the product was not an anhydro-derivative of I, which should exhibit strong absorbance in that

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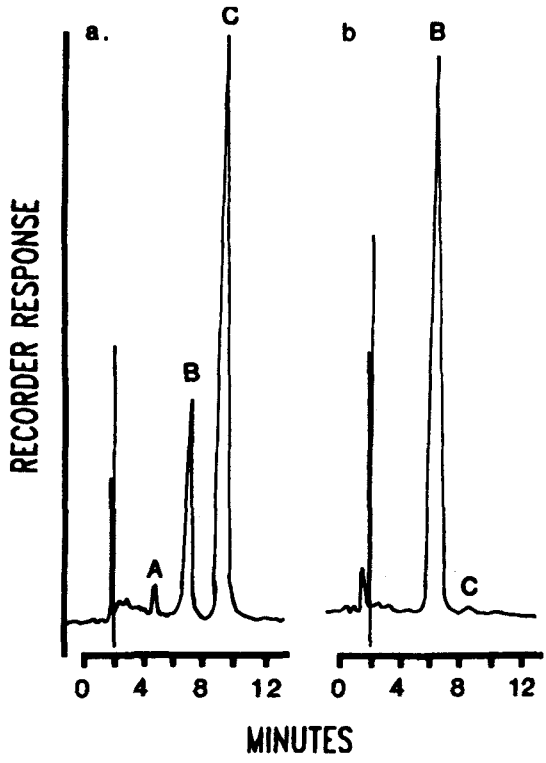

Figure 3: Chromatograms of mixture produced by heating I in glacial acetic acid for 2 hr on steam bath (a) and 4-epi-I after preparative HPLC separation from the above mixture, (b) Peak Identification and chromatographic conditions as in Fig. 2.

region(5). Also, the 4-epimers of tetracycline analogs, in general, have been shown to have less UV absorbance in the 250-300 nm region than the parent compound(5). This is to be expected since C-4 epimerization changes the conformation of the ß-dicarbonyl system of ring A which contributes to the strong absorption at around 260 nm.

Several TLC methods for tetracyclines and their breakdown products reported in the literatuae were tried, but only one was applicable to the resolution of I and its 4-epimer (9). This

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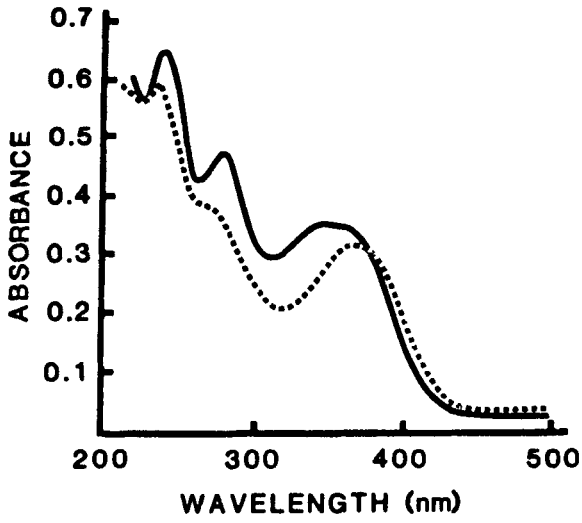

Figure 4: Ultraviolet spectra of I (–) and 4-epi-I (--) in the HPLC mobile phase (0.001 M ammonium edetate, pH 6.6-tetra hydrofuran, 87:13, v/v pH adjusted with glacial acetic acid) collected by preparative HPLC. Concentrations were 16.6 µg/ml for I and 15.1 µg/ml for 4-epi-I.

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method prescribed silica gel plates soaked in disodium edetate (0.1 M) for 1 hr and activated by drying overnight at room temperature. To shorten the time for analysis, the activation was modified to drying the plate with a warm-air dryer for a minimum of 1 hr and to using high-performance TLC plates with a preadsorbent application layer instead of standard silica gel plates. Development of the plate in methyl ethyl ketone saturated with McIlvaine's buffer at pH 4.7 gave a very efficient resolution of I (R_f ~ 0.24) and II (R_f ~ 0.09).

It has been documented that the circular dichroism (CD) curves of tetracycline and 4-epitetracycline show a large difference in molar ellipticity between the two epimers at 262 nm(16). The CD-data for I and II demonstrated the same (Fig. 5). The large negative molar ellipticity of I, due to

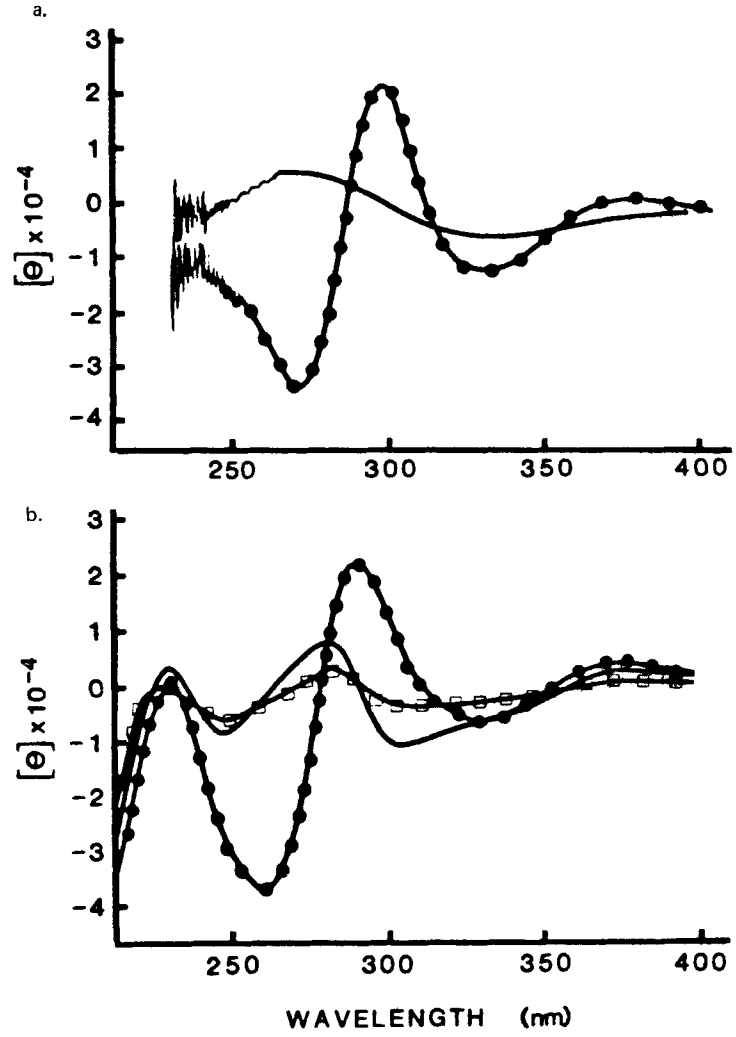

Figure 5: Molar ellipticities (Θ) of I (●) and synthetic 4-epi-I (-) in the HPLC mobile phase (the same as in Fig. 4) (a) and I (●), synthetic 4-epi-I (-) and the 4-epi-I isolated from aged MCSS cream (□) in methanolic-hydrochloric acid (0.01 N) (b) as function of wavelength. Concentrations were 28 µg/ml for I and for 4-epi-I.

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the A-ring band at around 260 nm, was almost nonexistent in both the synthetic 4-epimer and in the 4-epimer isolated from the formulation of I.

The overall data conclusively point to the presence of 4-epimer of I as the major degradation product in the pharmaceutical formulations of I under the stress conditions. In addition, the data suggest that the epimerization at C-4 is not the favored reaction, but rather an equilibrium state which favors the intact meclocycline.

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