Liquid Chromatographic Analysis of Ethacrynic Acid and Degradation Products in Pharmaceutical Systems

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Abstract □ An accurate, reproducible, and specific reversed-phase high-performance liquid chromatographic (HPLC) system was developed for the determination of ethacrynic acid and its degradation products. The method was used in stability studies of the drug in the solid state, in solution, and in dosage forms. Three degradation products were isolated by preparative chromatography and identified by several techniques, principally NMR and MS. TLC R_f and HPLC response factors are quoted. A degradation scheme consistent with the observed stability profiles is proposed.

Ethacrynic acid (**1**) is a phenoxyacetic acid having rapid diuretic action. Early work by Cohen,[1] who analyzed ethacrynic acid using a polarographic technique, identified the principal route of degradation in tablets and lyophilized injections as a Diels–Alder type of condensation leading to the formation of a dimer (**2**), [4-[2-[4-(carboxymethoxy)-2,3-dichlorobenzoyl]-3,4-dihydro-2,5-diethyl-2*H*-pyran-6-yl]-2,3-dichlorophenoxy]-acetic acid. This polarographic procedure was suitable for the determination of ethacrynic acid in the presence of several of its reaction products, including **2**. Hagerman and co-workers,[2] primarily investigating the conditions required for successful manufacture of a lyophilized product, determined both ethacrynic acid and **2** by a high-performance liquid chromatographic (HPLC) procedure using gradient elution. Other workers[3] using HPLC analysis have confirmed this dimer as the principal degradation product in concentrated aqueous solutions and calculated the kinetics of this reaction as a function of temperature and concentration.

Goerlitzer and Hoebbel,[4] investigating the reactions of **1** with sodium hydroxide, isolated and identified one of the products (2,2′-[(2,4-diethyl-1,5-dioxo-1,5-pentanediyl)bis[(2,3-dichloro-4,1-phenylene)oxy]]bisacetic acid; **5**) and proposed a reaction scheme involving [2,3-dichloro-4-(2-hydroxymethyl-1-oxobutyl)phenoxy]acetic acid (**3**) as an unstable intermediate. Compound **5** was identified almost simultaneously by Auterhoff and Thinnes.[5]

The present report investigates the stability of ethacrynic acid in dilute buffered solutions and reveals the presence of several degradation products for which documented analytical methods had inadequate specificity.[3,6–9] The paper describes a rapid, reversed-phase HPLC procedure to determine ethacrynic acid and its degradation products, three of which are isolated and identified. The stability of the drug in dilute aqueous solution and in dosage forms is investigated.

Experimental Section

Materials—Ethacrynic acid conformed to USP requirements and dimer **2** and **4** were used as received (Merck Sharp & Dohme, West Point, PA). Methanol (HPLC grade) and deionized water were filtered through a 0.22-μm filter prior to use. All other chemicals and reagents were of analytical grade.

Apparatus—The HPLC (model 1084B, Hewlett-Packard Ltd., Workingham, England) was equipped with a gradient elution facility, a variable-wavelength UV detector, an automatic sampler, injector, and fraction collector. Peak areas were measured by electronic integration.

Preparative Chromatography—Degradation product **3** was generated in a 5% (w/v) slurry of **1** in 0.1 M HCl. Compounds **4** and **5** were generated in a 5% (w/v) solution of **1** in 1 M NaOH. After storage at 60°C for 30 d, the solutions were adjusted to pH 6 with an HCl or NaOH solution, concentrated by evaporation under reduced pressure to about a quarter of the initial volumes, and then diluted approximately twofold with methanol to give clear solutions which were chromatographed.

The mobile phase was a mixture of methanol and 0.1 M ammonium acetate adjusted to pH 6 with glacial acetic acid. The column (250 × 10 mm) packed with Hypersil 5-μm, ODS (Shandon Southern Products, Runcorn, Cheshire, England), was equilibrated at 50°C with a mobile phase containing 20% methanol by volume. Chromatography was carried out on multiple 200-μL injections of the degraded sample solutions, using a flow rate of 3.0 mL/min, detection at 290 nm, recorder sensitivity of 2 AUFS, and a linear gradient of 20–50% methanol by volume in 20 min. The fractions corresponding to the three principal degradation products **3**, **4**, and **5** eluting after 5, 9, and 16 min, respectively, were collected automatically and freeze-dried.

Identification and Characterization of Degradation Products—The purity of each freeze-dried degradation product was assessed by HPLC and TLC, before and after recrystallization. Identities were established by ^1H or ^{13}C NMR (model AM 250, FT NMR Spectrometer, Bruker Spectrospin Ltd., Coventry, England), by MS (model 16F Mass Spectrometer, V. G. Micromass, Altrincham, Cheshire, England) Spectrometer, Hewlett-Packard Ltd.), IR (model 781 Infrared Spectrophotometer, Perkin Elmer Ltd., Beaconsfield, Bucks, England), and by melting point determination. UV spectra (model 8450A Photodiodearray Spectrometer, Hewlett-Packard Ltd.) were recorded, using the analytical HPLC mobile phase as solvent.

[2,3-Dichloro-4-(2-hydroxymethyl-1-oxobutyl)phenoxy]acetic Acid (3)—The crude, freeze-dried product (70 mg of white powder; 92% by HPLC at 278 nm and containing 7% **4**) was not purified by recrystallization for reasons of low yield and poor chemical stability. ^1H NMR (acetone-d_6): δ 0.91 (t, 3, CH_2CH_3), 1.57, 1.73 (m, 2, $CHCH_2CH_3$), 3.36 (m, 1, CHCO), 3.68, 3.82 (m, 2, $CHCH_2OH$), 4.98 (s, 2, OCH_2COOH), 7.13 (d, 1, Ar H-5), and 7.60 ppm (d, 1, Ar H-6); ^{13}C NMR (acetone-d_6): δ 11.90 (CH_3CH_2), 22.23 (CH_3CH_2), 55.62 ($CH_2CH(CO)CH_2$), 63.14 ($HOCH_2CH$), 66.5 (OCH_2), 112.14 (Ar C-6), 123.38 (Ar C-2), 128.66 (Ar C-5), 131.24 (Ar C-3), 135.27 (Ar C-4), 157.07 (Ar C-1), 169.18 (COOH), and 204.15 ppm (ketone CO); MS (methyl ester; source temperature, 200°C; ionizing energy, 70 eV): m/z 304 ($M - CH_2O$), 261, and 243. No molecular ion was seen. IR (nujol): 1700 (ketone), 1740

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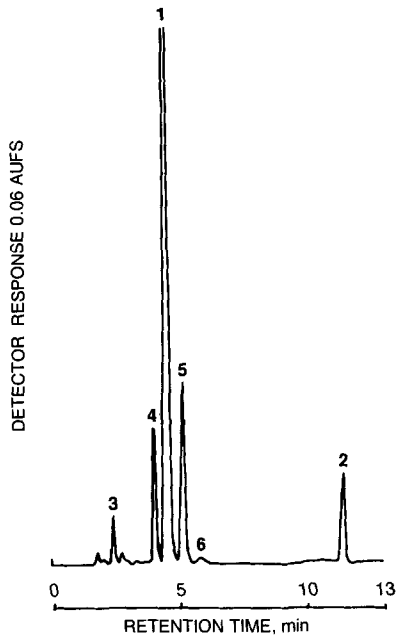


Figure 1—*Chromatogram of a test mixture of ethacrynic acid (1) and added degradation products* **2–6**.

(COOH), 3450 cm^{-1} (OH); UV: λ_{max} 265 nm, (log ϵ 3.90).

[2,3-Dichloro-4-(1-oxobutyl)phenoxy]acetic Acid (4)—The crude, freeze-dried product (160 mg; 96% by HPLC) was recrystallized from cyclohexane:*tert*-butyl methyl ether (70:30) to give 110 mg of white crystals. The melting point was in good agreement with the reported value;[10] ^1H NMR and IR were identical with data obtained on authentic material (Merck Sharp & Dohme). Elemental analyses were within ±0.4% of the theoretical value; UV: λ_{max} 267 nm (log ϵ 3.919).

2,2′ - [2,4 - Diethyl - 1,5 - dioxo - 1,5 - dioxo - 1,5 - pentanediyl)bis-[(2,3-dichloro-4,1-phenylene)oxy]]bis-acetic Acid (5)—The crude, freeze-dried product (470 mg; 97% by HPLC) was recrystallized from methanol to give 280 mg of white crystals. ^1H NMR, MS, IR, and mp were in excellent agreement with reported data.[4,5] Elemental analyses were within ±0.6% of the theoretical value; UV: λ_{max} 272 nm (log ϵ 4.265).

Analytical Liquid Chromatography—*Conditions*—The aqueous mobile phase component was phosphate buffer, prepared by adjusting 0.05 M phosphoric acid to pH 5.6 with concentrated NaOH solution. The column (250 × 4.5 mm) packed with Hypersil 5-μm, ODS (Shandon Southern Products), was equilibrated with 52% methanol:buffer by volume. Chromatography was carried out at 50°C using a flow rate of 1.5 mL/min, detection at 278 nm, a recorder sensitivity of 0.06 AUFS, and 20-μL injection volumes. Elution proceeded for 6 min at 52% methanol, then for 7 min at 65% methanol. The column was allowed to re-equilibrate for 5 min at starting composition. As an indication of the system suitability,[11,12] plate count, capacity factor, and tailing factor were measured using the ethacrynic acid peak. Comparability of results over long time periods and among different columns was ensured by chromatographing a test solution containing ethacrynic acid and known degradation products (Fig. 1). Peak areas averaged from at least three determinations were used in subsequent calculations.

Standard and Sample Solutions—Standard solutions of ethacrynic acid were accurately prepared in 50% methanol in water (v/v). A series of dilutions covering the 1–1000 μg/mL range was made to determine sensitivity and linearity of response. For assays and degradation studies, solutions of 50 μg/mL were used.

Table I—Chromatographic Data of Ethacrynic Acid (1) and Degradation Products 2–6

Component	HPLC		TLC	
	Relative Retention Time	Response Factor at 278 nm	R_f	Relative R_f
1	1.00	1.00	0.45	1.00
2	2.50	1.08	0.34	0.76
3	0.53	0.54	0.23	0.51
4	0.85	0.52	0.42	0.93
5	1.14	0.48	0.27	0.60
6	1.30	—	—	—

Samples of 10 tablets (Edecrin, 50 mg, BN 20871, Merck Sharp & Dohme Ltd.) were allowed to disintegrate in 500-mL volumetric flasks each containing 250 mL of water. The slurries were made to volume with methanol, sonicated, and portions were centrifuged. Aliquots (5.0 mL) of the clear supernatant were diluted to 100 mL with 50% methanol in water (v/v), and the solutions were chromatographed.

Individual lyophilized injections (Edecrin for injection, 50 mg, BN 08401, Merck, Sharp & Dohme Ltd.) were reconstituted with 50% methanol in water (v/v) and diluted to 50 mL. Aliquots (5.0 mL) were diluted to 100 mL with the same solvent and the solutions were chromatographed.

Solutions for Kinetic Studies—For each solution, ~12.5 mg of ethacrynic acid was weighed accurately into a 250-mL conical flask and warmed to the required temperature. A 0.02 M acetate:borate:phosphate buffer[13] (250 mL) adjusted to selected pH values and warmed to the appropriate temperature were added to each flask. The drug was dissolved, the solution was mixed, and the flasks were stored in constant temperature ovens. Aliquots were withdrawn at intervals, rapidly cooled to room temperature, and immediately analyzed by HPLC.

HPLC response factors at 278 nm were calculated chromatographically by assay of **2** and the isolated degradation products, using ethacrynic acid as external standard. Peak areas of the degradation products were corrected to a weight basis by multiplying by the appropriate response factor and recalculating as a percentage. In degraded solutions, peak homogeneity was confirmed by rapid UV scanning of leading and tailing edges and by subsequent superimposition of normalized spectra. No spots additional to the number of HPLC peaks were resolved by TLC, which was carried out as follows: solutions were adjusted to pH 2 with HCl and extracted with small volumes of ether. Aliquots of the ether extracts were applied to silica plates, the plates were developed using toluene:glacial acetic acid:ethanol (75:20:5 by volume), and spots were visualized by fluorescence quenching under short-wavelength UV light. HPLC and TLC chromatographic data are listed in Table I.

Results and Discussion

Initial HPLC development was aimed toward determination of ethacrynic acid and its dimer **2** using a rapid reversed-phase isocratic system giving acceptable resolution and sensitivity. However, the observation that some stressed solutions contained additional peaks with retention characteristics similar to ethacrynic acid called for a system capable of resolving these degradation products. Acceptable isocratic chromatograms could not be obtained using C_{18}, C_8, C_3-nitrile, and C_3-amino bonded stationary phases in conjunction with mobile phases of methanol, ethanol, or acetonitrile buffered at pH 2.7–7, either in the presence or absence of ion-pairing agents such as tetra-*n*-butylammonium phosphate and tetramethylammonium phosphate. Resolution of all components (Fig. 1) could be achieved only at the sacrifice of sensitivity for the dimer **2**, that had an excessively long retention time. In isocratic chromatograms of the severely degraded drug, no peaks were seen

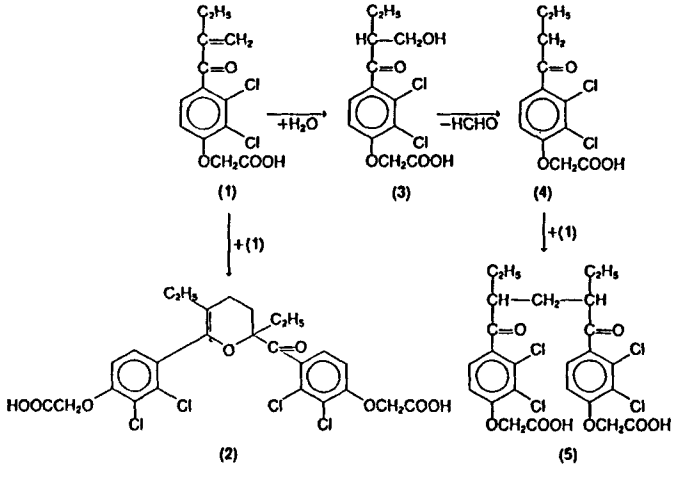
to elute between **6** and **2**, thereby allowing a choice between stepped isocratic elution in which **2** was eluted rapidly as a sharp peak following **6**, or gradient elution. The former was selected since it offered the advantages of the greater stability of isocratic systems. Baseline disturbances arising from the sudden mobile phase change were found to be minimal because of the high detection wavelength. The conditions finally adopted are tolerant of some variation in pH, ionic strength, and nature of buffer provided that the minimum plate count for ethacrynic acid is 15,000, the tailing factor is not more than 1.5, and that the capacity factor is maintained at ~3 by adjusting the methanol content of the mobile phase. The optimum pH range for chromatography is between 5 and 6. At lower and higher pH, resolution of **4**, **5**, and **6** deteriorates. Compounds **4** and **1** may coelute if the column temperature is altered by ±5°C. Several octadecyl-bonded stationary phases meeting the systems suitability criteria have been used successfully and, in general, only minor changes in the percent methanol are required.

The response factors listed in Table I are averages of values calculated on several occasions over a long time period and on different HPLC instruments of the same model. No detection wavelength is ideal, because of the dissimilarity of UV absorption characteristics of ethacrynic acid and its degradation products. Accurate response factors were determined routinely using a test solution containing known amounts of degradation products.

The chromatographic precision of the system, determined on peak areas of triplicate injections and expressed as RSD, was 0.45% for solutions containing ethacrynic acid in the 0.025–0.25 $mg \cdot mL^{-1}$ range and was <1.3% in the 0.01–1.0 $mg \cdot mL^{-1}$ range. The detector response was linear over the wider of these concentration ranges, with a regression coefficient of 0.9999. The limit of detection was ~4 ng. Assays of commercial tablet and injection formulations gave results within 1.2% of label claim. Recoveries of added ethacrynic acid were 100 ± 0.4%.

The mixed acetate–borate–phosphate buffer used in solution studies provided satisfactory buffering capacity at equal anion concentration over the pH range of 2–12. Sodium was chosen as the cation to avoid a specific incompatibility reported between ethacrynic acid and ammonium ion.[6] Low solubility of ethacrynic acid in acidic aqueous solution severely restricted the maximum concentration that could be employed, if comparability of results over the entire pH range were to be maintained. At the concentration adopted, the drug and its degradation products remained in solution and sensitivity of detection was adequate.

The α,β-unsaturated ketone group in ethacrynic acid is chemically reactive. This is exploited in the polarographic and bromination tablet assays of the US and British Pharmacopoeias[7,8] and is confirmed by the present investigations in which several degradation pathways were seen (Scheme I). In the presence of water, the initial reaction of ethacrynic acid involves hydration of the methylene double bond to form **3** at a rate which is temperature- and pH-dependent, increasing as the pH is raised from 2 to 12 (Fig. 2). In dilute solutions of low pH, **3** was the only significant degradation product detected during the period



Scheme I—Degradation products of ethacrynic acid.

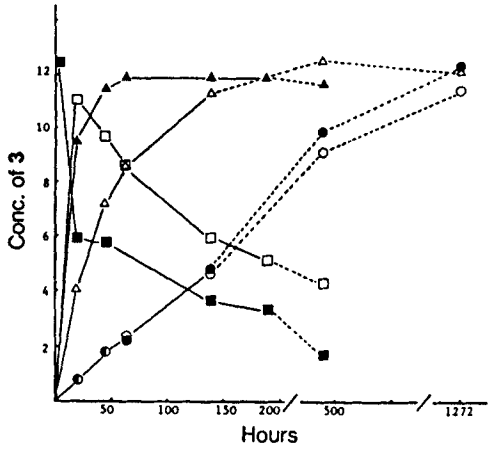


Figure 2—*Influence of pH on the formation of degradation product 3, as percent (w/w) of initial ethacrynic acid, in buffered solutions of ethacrynic acid (0.05 mg/mL) stored at 60°C. Key: (○) pH 2; (●) pH 4; (△) pH 6; (▲) pH 8; (□) pH 10; (■) pH 12. Dotted lines between points reflect breaks in the abscissa.*

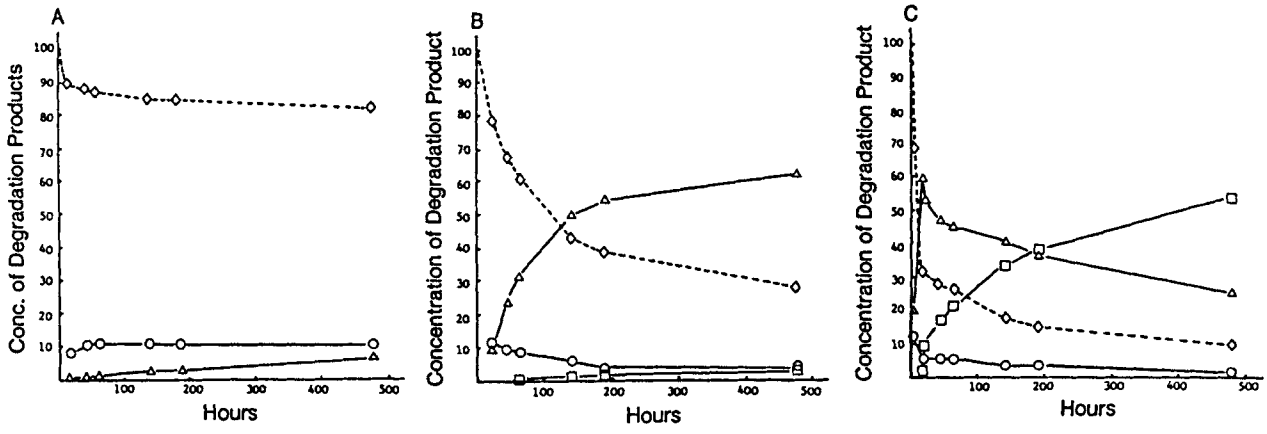


Figure 3—*Degradation of ethacrynic acid (0.05 mg/mL), as percent (w/w) of initial ethacrynic acid, in buffered aqueous solutions stored at 60°C at pH 8 (A), pH 10 (B), and pH 12 (C). Key: (◇) ethacrynic acid (1); (○) 3; (△) 4; (□) 5.*

studied, but in aqueous slurries of ethacrynic acid and in the solid state, a Diels–Alder type of condensation takes place, generating **2**. In strongly alkaline solutions the rate of hydration is rapid but the amounts of **3** found are limited by a subsequent reaction in which formaldehyde is eliminated from **3**, giving rise to **4**. Similarly, the levels of **4** reached were limited by the involvement of **4** in a Michael-addition reaction[14] with ethacrynic acid giving rise to **5** (Fig. 3).

In severely stressed alkaline solutions additional degradation products (**6** and an HPLC peak at relative retention time 2.9) were observed at low levels. No attempts were made to isolate and identify these degradation products, but UV spectra and HPLC retention characteristics suggest that **6** resembles **5**.

Degradation of ethacrynic acid to **2** occurs chiefly in aqueous slurries and in solid dosage forms when stored at 60°C for long periods. Small amounts of **3** were found in the lyophilized product but the levels did not increase on storage, indicating that this reaction product is an occasional degradation product arising during manufacture. The stability of both the neat drug and the dosage forms was similar to that reported by Cohen[1] and Hagerman et al.[2] The virtual absence of **2** in stressed dilute solutions can be attributed to concentration dependence of this dimerization reaction, the kinetics of which have been reported.[3] Kinetics of the remaining reactions in dilute solution are under investigation.

The presence of the α,β-unsaturated ketone moiety is a major contributor to the biological activity of ethacrynic acid and its analogues.[15] The loss of the methylene olefinic bond noted in all the degradation products identified, therefore, would be expected to result in significantly reduced biological activity.

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