Journal of Pharmaceutical & Biomedical Analysis
Vol. 5, No. 4, pp. 369–378, 1987
Printed in Great Britain

0731–7085/87 $3.00 + 0.00
Pergamon Journals Ltd.

The influence of the ammonium ion on the stability of ethacrynic acid in aqueous solution

R. J. YARWOOD,[1,2] W. D. MOORE[2]* and J. H. COLLETT[3]

[1] *Wyeth Laboratories, Huntercombe Lane South, Taplow, Berks SL6 0PH, UK*
[2] *Merck Sharp and Dohme Research Laboratories, Hoddesdon, Hertfordshire EN11 9BU, UK*
[3] *Department of Pharmacy, University of Manchester, Manchester M13 9PL, UK*

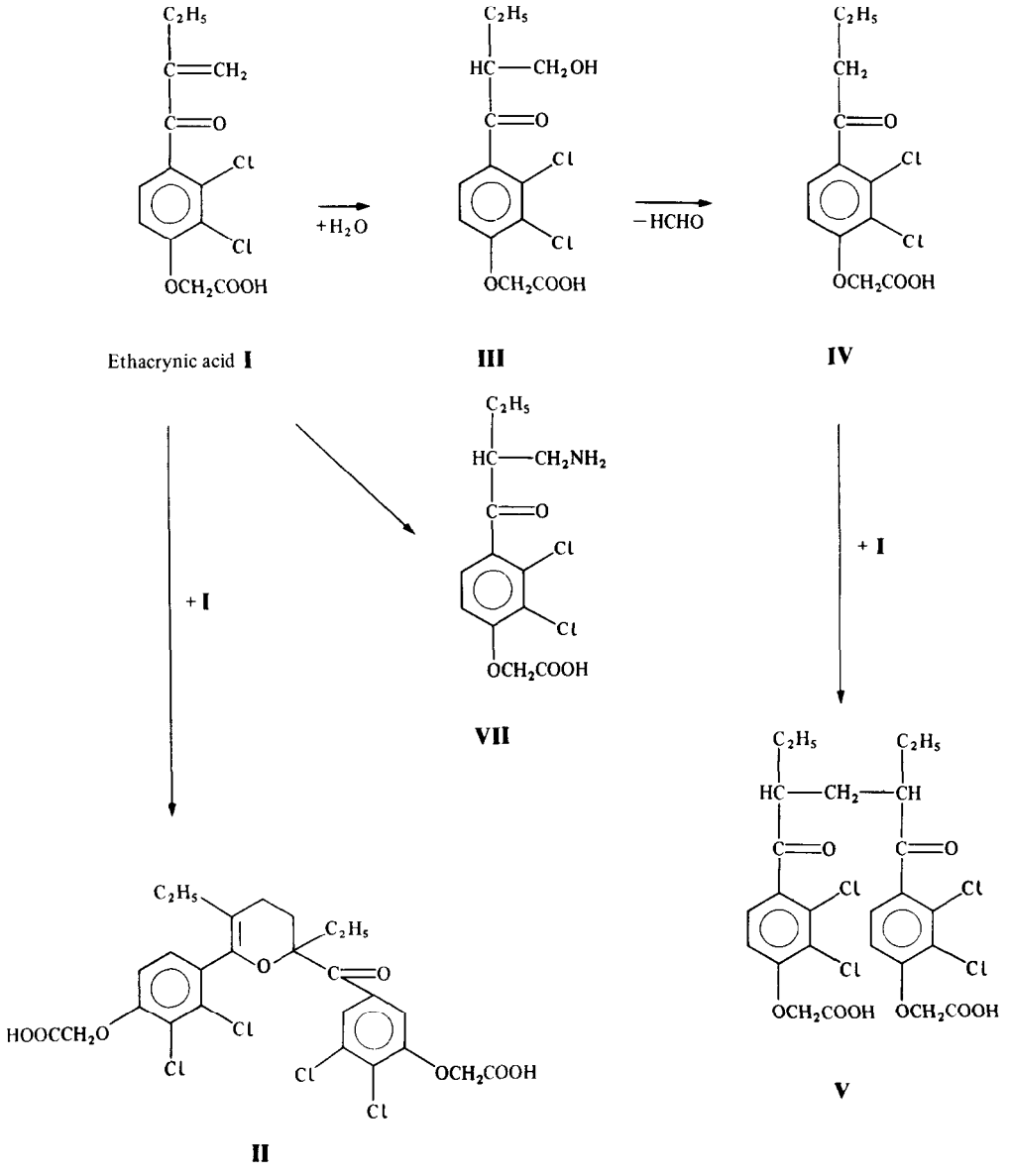
Abstract: Investigations by reversed-phase HPLC into the stability of ethacrynic acid in buffered aqueous solutions containing either sodium or ammonium ions showed that the extent of degradation was influenced both by the species and concentration of the cation. A reported incompatibility between ethacrynic acid and the ammonium ion, attributed to the influence of the ammonium ion on an equilibrium existing between ethacrynic acid and one of its known degradation products, was shown to be due to the generation of an additional degradation product in ammonium-containing solutions only. This product was isolated and identified. Different pathways of degradation were shown to be operative in sodium- and ammonium-containing solutions. The addition of formaldehyde or a formaldehyde scavenger (hydroxyammonium chloride) was found to influence the rate of loss of ethacrynic acid, but the decomposition products provided no evidence for the existence of the reported equilibrium.

Keywords: *Ethacrynic acid; reversed-phase high-performance liquid chromatography; stability; incompatibility.*

Introduction

The stability of the diuretic compound ethacrynic acid in aqueous solutions containing sodium ions has previously been investigated [1, 2]. Scheme 1 shows the products of degradation (II–V) and the deduced pathways of decomposition for both dilute and concentrated solutions of the drug. Recent work by Das Gupta [3] has highlighted a specific concentration-dependent incompatibility of ethacrynic acid with ammonium-containing solutions, leading to decreased stability relative to ammonium-free solutions. Three of the degradation products previously identified by the present authors in solutions containing sodium as the only cation were suggested by Das Gupta to be products of the specific incompatibility with the ammonium ion and to exist in equilibrium with ethacrynic acid. The author suggested that formaldehyde was one of the decomposition products and supported the conclusions by observing the effect of added formaldehyde or hydroxyammonium chloride (the latter serving as a formaldehyde scavenger) on the reaction equilibrium of the degradation of ethacrynic acid. No other

*To whom correspondence should be addressed.


C2H5
C=CH2
C=O
Cl
Cl
OCH2COOH
+H2O
HC—CH2OH
−HCHO
CH2
Ethacrynic acid I
III
IV
+ I
HC—CH2NH2
VII
HC—CH2—CH
HOOCCH2O
II
V

evidence was presented concerning the structures of the postulated degradation products. The present study using reversed-phase high-performance liquid chromatography (HPLC) was undertaken to investigate the rates and products of the degradation of ethacrynic acid in solutions containing ammonium ions and to compare the results with those already established for solutions containing sodium ions.

Experimental

Materials

Ethacrynic acid conformed to USP specifications and sodium ethacrynate and ethacrynic acid dimer (II) were used as received (Merck Sharp and Dohme, West Point, PA). [4-(2-*aminomethyl*-1-*oxobutyl*)-2,3-*dichlorophenoxy*] *acetic acid* (VII) was prepared and characterized as described below. Degradation products III, IV and V were isolated previously by preparative HPLC [1]. Methanol (HPLC grade) and deionized water were filtered through a 0.22 μm membrane filter before use. All other chemicals were of analytical reagent grade.

Apparatus

The HPLC instrument (Model 1084B, Hewlett–Packard Ltd., Wokingham, England) was equipped with a gradient elution facility, a variable wavelength UV detector, an automatic sampler and injector. Peak areas were measured by electronic integration. The column (250 × 4.5 mm i.d.) was packed with Hypersil 5μ, ODS (Shandon Southern Products, Runcorn, Cheshire, England).

High-performance liquid chromatography

The aqueous mobile phase component was phosphate buffer, prepared by adjusting 0.05 M potassium dihydrogen phosphate to pH 5.4 with 5 M sodium hydroxide. The column was equilibrated with methanol–aqueous buffer (42:58, v/v). 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 an injection volume of 20 μl. Elution proceeded for 9 min with 42% methanol in buffer as the mobile phase, then for 6 min with 60% methanol as buffer. The column was then allowed to re-equilibrate for 5 min with the former mobile phase. System suitability [4, 5] was evaluated by calculating the number of theoretical plates, capacity factor and symmetry factor of the ethacrynic acid peak. The comparability of results over long time periods and with different columns was ensured by routinely chromatographing a standard solution of ethacrynic acid and its degradation products (II, III, IV, V and VII) (Fig. 1).

Preparation of solutions

Standard solutions of ethacrynic acid were accurately prepared in 50% methanol in water (v/v). A series of dilutions covering a range of 1–1000 μl/ml was made to determine the sensitivity and linearity of the response. For assays and degradation studies, solutions of 50 μg/ml were used. The average of at least three determinations was used in subsequent calculations. Chromatographic response factors for degradation products III, IV, V and VII were obtained using 10 μg/ml solutions.

For kinetic studies, an aqueous stock solution of sodium ethacrynate containing the equivalent of 10 mg/ml ethacrynic acid was prepared. Aliquots of the stock solution were diluted with the appropriate buffer to contain 0.5 mg/ml ethacrynic acid. An aqueous

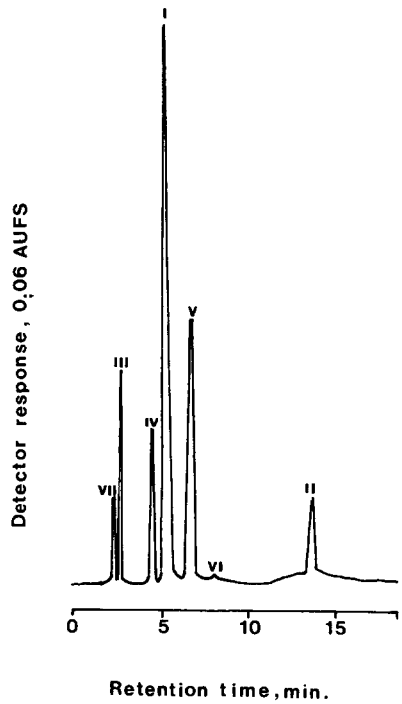


Figure 1
HPLC chromatogram. Test mix containing ethacrynic acid and known degradation products (II, III, IV, V, VI and VII).

Table 1
pH of solutions prepared for kinetic studies

Solution	Initial pH (20°C)
1. Disodium hydrogen phosphate 0.02 M	8.1
2. Disodium hydrogen phosphate 0.04 M	8.2
3. Diammonium hydrogen phosphate 0.02 M	7.7
4. Diammonium hydrogen phosphate 0.04 M	7.8
5. Disodium hydrogen phosphate 0.04 M, formaldehyde 0.1 M	8.2
6. Diammonium hydrogen phosphate 0.04 M, formaldehyde 0.1 M	5.9
7. Diammonium hydrogen phosphate 0.04 M, formaldehyde 0.1 M, ammonium hydroxide to adjust pH	7.5

solution was also prepared and immediately assayed by HPLC in order to establish the initial concentration of the solutions used in the degradation studies. The buffers used, their final concentrations, and their pH values are listed in Table 1. All solutions were stored in sealed flasks at 60°C in a constant temperature oven. Aliquots were withdrawn at intervals, cooled rapidly to room temperature and analyzed immediately by HPLC. Peak areas of degradation products were corrected to an equivalent weight of ethacrynic acid by multiplying by the appropriate response factor and recalculating as a percentage.

The following solutions were used to investigate the influence of hydroxyammonium chloride on the degradation of ethacrynic acid:

(i) Ethacrynic acid (25.0 mg) was dissolved in and made up to 50 ml with 0.25 M ammonium hydroxide solution;

(ii) Ethacrynic acid (25.0 mg) was dissolved in and made up to 50 ml with 0.25 M ammonium hydroxide solution containing hydroxyammonium chloride (2%, w/v);
(iii) Solution prepared as (ii) but ammonium hydroxide was replaced with 0.25 M sodium borate buffer (pH 9.5).

These solutions were refluxed for 1 h, cooled to room temperature, neutralized with 0.1 M sulphuric acid, made up to 500 ml with water and analyzed by HPLC.

Isolation and characterization of compound VII

Compound VII was synthesized by the reaction of ethacrynic acid with ammonia and isolated as described below. Its identity was established by nuclear magnetic resonance spectrometry (Model AM 250, FT NMR Spectrometer, Bruker Spectrospin Ltd., Coventry, England) operating in the ^{1}H and ^{13}C modes; by mass spectrometry (Model 16F Mass Spectrometer, V.G. Micromass, Altrincham, Cheshire, England) and by infrared spectrometry (Model 781 Infrared Spectrometer, Perkin–Elmer Ltd., Beaconsfield, Bucks, England). Purity was assessed by potentiometric titration (Model 636 Titroprocessor, Metrohm, Herisau, Switzerland) and by HPLC. Ultraviolet spectra (Model 8450A Diodearray Spectrophotometer, Hewlett–Packard Ltd., Winnersh, England) were recorded in methanol–phosphate buffer, 0.05 M, pH 5.4 (42:58, v/v). The equivalence of isolated VII (crude, purified and the recrystallized salt) to the HPLC peak of VII obtained with solutions of ethacrynic acid degraded in ammonium-containing buffers was demonstrated by spiking and chromatographing, using, in addition to the HPLC conditions already described, a linear gradient of 30% to 80% methanol in 20 min.

[4-(2-*aminomethyl*-1-*oxobutyl*)-2,3-*dichlorophenoxy*] *acetic acid* (VII). Ethacrynic acid (3.0 g) was dissolved in ammonia solution (100 ml, 5% w/v) and allowed to stand at room temperature for 18 h. The solution was acidified to ~pH 1 with hydrochloric acid (35% w/w), cooled in ice-water for 1 h then filtered. The pH of the clear filtrate was raised slowly by the addition of sodium hydroxide solution (10% w/v), until a white precipitate formed at ~pH 5. The slurry was cooled in ice-water and centrifuged. The supernatant liquid was discarded and the precipitate was repeatedly suspended and washed with cold water until the washings gave a negative reaction for chloride, using silver nitrate and nitric acid. The residue was dried at 80°C under vacuum to give crude VII (2.1 g; 97.5% by HPLC at 272 nm and containing 1.6% ethacrynic acid).

Attempts to recrystallize compound VII from neutral solvents were unsuccessful, because of its very low solubility in the common organic solvents and in water. Further purification was achieved by finely grinding the crude VII (2.0 g) in a mortar, dispersing in methanol (20 ml) and shaking for 24 h. The suspension was centrifuged, the sediment washed with methanol and dried at 60°C under vacuum to give purified VII as a white powder (1.8 g; 99.0% by HPLC at 272 nm and containing 0.6% ethacrynic acid, 0.2% III and 0.1% IV). ^{1}H NMR (methanol-d_4 + DCl): δ 0.89 (t, 3, $C\underline{H}_3CH_2$), 1.61, 1.75 (m, 2, $CH_3C\underline{H}_2$), 3.86 (m, 1, $CH_2C\underline{H}CH_2$), 3.18, 3.40 (m, 2, $C\underline{H}_2NH_2$), 4.92 (s, 2, $OC\underline{H}COOH$), 7.13 (d, 1, Ar $\underline{H}$-5) and 7.72 ppm (d, 1, Ar H-6). ^{13}C (DMSO-d_6): δ 10.16 ($\underline{C}H_3$), 22.28 ($CH_3\underline{C}H_2$), 37.71 ($\underline{C}H_2\ NH_2$), 48.20 ($\underline{C}HCO$), 65.66 ($O\underline{C}H_2$), 111.50 (Ar $\underline{C}$-6), 121.98 (Ar $\underline{C}$-4), 128.64 (Ar $\underline{C}$-5), 130.32 (Ar $\underline{C}$-2), 131.22 (Ar $\underline{C}$-3), 156.44 (Ar $\underline{C}$-1), 168.81 ($\underline{C}OOH$), and 200.47 (ketone $\underline{C}O$). MS (disilylated with *N,O*-bis(trimethylsilyl)-acetamide in dimethylformamide, 1:1, 10 min reaction at room temperature; ionizing energy 70 eV, source temperature 200°C): *m/z* 463 (M^+), 448 ($M-CH_3$), 428

(M–Cl), and 434, 374, 339, 319, 243. IR (nujol): ν_{max} 3200–3100 and 1630 (NH), 1710 (ketone) and 1740 cm (COOH). UV: λ_{max} 222 nm (ϵ 17190), 272 nm (ϵ 9100); equivalent weight (EW) (perchloric acid titration) 321; m.p. 192°C (decomp.).

Analysis. Found: C, 48.6; H, 4.72; Cl, 22.18; N, 4.34. $C_{13}H_{15}Cl_2NO_4$ requires C, 48.77; H, 4.72; Cl, 22.14; N, 4.38.

VII was recrystallized as the hydrochloride salt: the purified amine (1.0 g) was slurred in acetonitrile (50 ml) and warmed to 50°C. The suspension was slowly titrated with hydrochloric acid (1.0 M, ~3.5 ml) until clear, then evaporated to dryness at 50°C under reduced pressure and dried at 60°C under vacuum. After recrystallization from ethanol and drying at 80°C under vacuum, the hydrochloride salt of VII was obtained as white crystals (0.8 g; 99.8% by HPLC at 272 nm). NMR (^1H and ^{13}C) and MS, carried out as described above, gave structural data identical to those obtained for VII. UV: λ_{max} 222 nm (ϵ 17200), 272 nm (ϵ 9095); EW (perchloric acid titration) 357; m.p. 195°C (decomp.); Cl^- (silver nitrate titration) 9.91%.

Analysis. Found: C, 43.6; H, 4.54; Cl, 29.6; N, 3.89. $C_{13}H_{16}Cl_3NO_4$ requires C, 43.78; H, 4.52; Cl, 29.82; N, 3.93.

Results and Discussion

Preliminary studies of the stability of ethacrynic acid in solutions containing the ammonium ion were carried out using the stepped, isocratic HPLC system previously described [1]. Poor resolution was observed between the known degradation product (III) and an unidentified degradation product (VII). Satisfactory resolution of these was achieved by reducing the methanol content of the mobile phase and slightly extending the analysis time (Fig. 1). The conditions finally adopted are tolerant of some variation in pH, ionic strength and nature of buffer provided that the minimum number of theoretical plates for ethacrynic acid is 12,000, the symmetry factor is not more than 1.5 and the capacity factor is maintained at ~4 by adjusting the methanol content of the mobile phase. The system was capable of detecting concentrations of ethacrynic acid down to 0.2 μg/ml and the detector response was found to be linear over the range 1–1000 μg/ml (n = 5; r = 0.9999). Degradation product VII was readily prepared by reaction of ethacrynic acid with ammonia solution and isolated by precipitation at ~pH 5. The response factor for purified VII relative to ethacrynic acid was found to be 0.52 at 278 nm; the values determined for degradation products II, III, IV and V were unchanged from those reported previously [1].

The rate of decomposition of ethacrynic acid is clearly dependent upon the cationic species and its concentration (Fig. 2). Initially the rate of loss of ethacrynic acid in ammonium-containing solutions was greater than in the corresponding sodium-containing solutions, but over longer term storage, the drug was shown to be more stable in the ammonium-containing solutions. Degradation in solutions containing sodium ions (Fig. 3) followed the pathway previously described [1] whereby ethacrynic acid is hydrated to form III which then loses formaldehyde, generating IV. IV subsequently reacts with ethacrynic acid to form V. VI, found only in severely degraded solutions, has not been identified. In ammonium buffers (Fig. 4) an additional degradation product (VII, Scheme 1) was generated rapidly, reaching levels dependent upon the cation concentration. Generation of VII accounts for the rapid initial loss of ethacrynic acid observed in ammonium solutions. Thereafter, the rate of degradation is slower in ammonium-

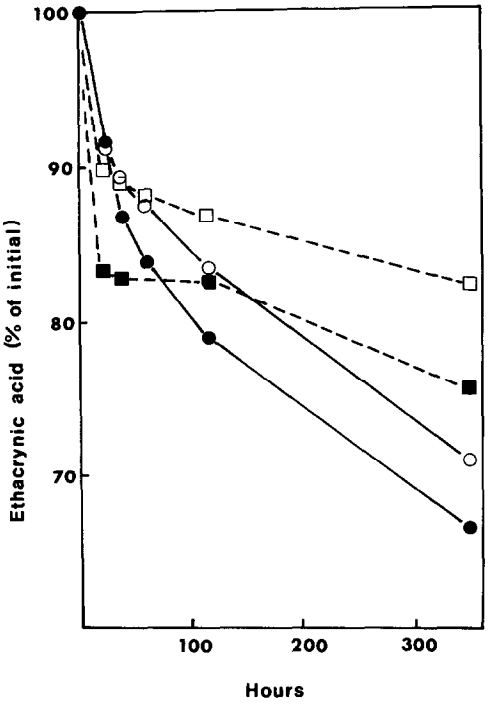


Figure 2
Stability of ethacrynic acid (0.5 mg/ml), stored at 60°C in selected buffer solutions. Key: □, Diammonium hydrogen phosphate 0.02 M; ■, Diammonium hydrogen phosphate 0.04 M; ○, Disodium hydrogen phosphate 0.02 M; ●, Disodium hydrogen phosphate 0.04 M.

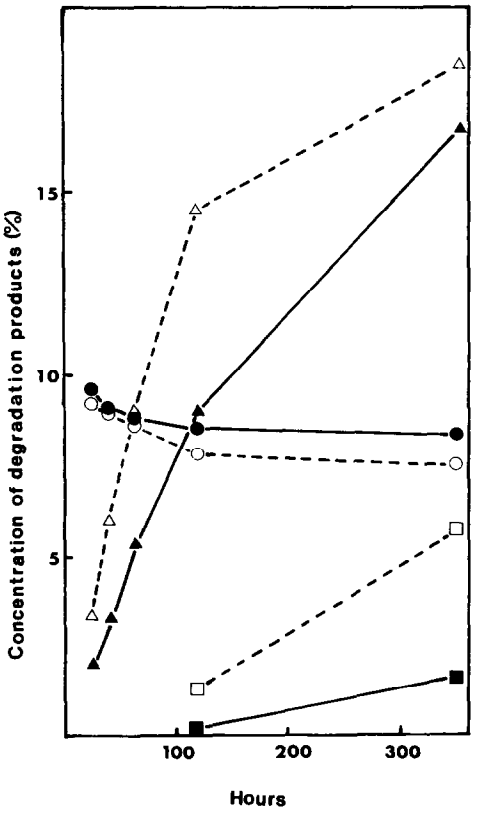


Figure 3
Degradation products generated from ethacrynic acid (0.5 mg/ml) in disodium hydrogen phosphate buffer solutions stored at 60°C. Key: Closed symbols, 0.02 M buffer; open symbols, 0.04 M buffer. ●, III; ▲, IV; ■, V.

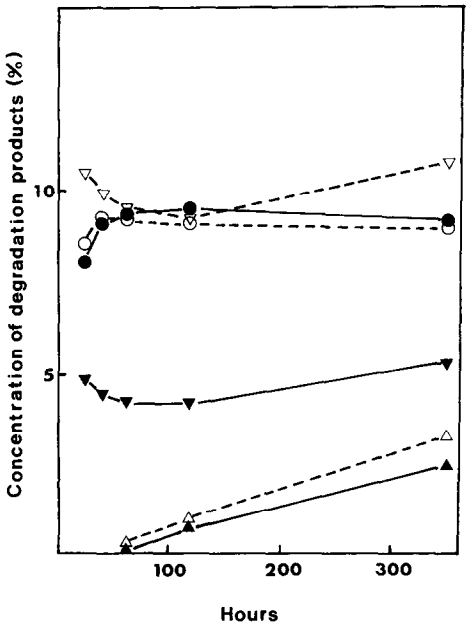


Figure 4
Degradation products generated from ethacrynic acid (0.5 mg/ml) in diammonium hydrogen phosphate buffer solutions stored at 60°C. Key: Closed symbols, 0.02 M buffer; open symbols, 0.04 M buffer. ●, III; ▲, IV; ▼, VII.

than in the sodium-containing solutions as would be anticipated from previous work on the pH-dependence of the rate of decomposition [1]. Small quantities of ethacrynic acid dimer (II) were found in those solutions stressed over the longest time periods.

Das Gupta, believing III and IV to be the products of the specific incompatibility in the ammonium-containing solution, suggested that the equilibrium of the reaction III → IV should be influenced by the addition of formaldehyde or of hydroxylamine hydrochloride (a formaldehyde scavenger) to the reaction solution [3]. These studies appeared to confirm this conclusion by the observation that added formaldehyde led to improved stability of ethacrynic acid in ammonium-containing solutions, whereas added hydroxylamine hydrochloride resulted in decreased stability. The observation in the present study that generation of VII was the principal early degradative pathway of ethacrynic acid in ammonium-containing solutions suggested that different mechanisms must be operating in the presence of formaldehyde or hydroxylamine hydrochloride.

The influence of added formaldehyde on the loss of ethacrynic acid in sodium- and ammonium-containing solutions is seen in Fig. 5. In sodium-containing solutions the stability of the drug is greater in the presence of formaldehyde although an additional polar degradation product is generated in small amounts. Chromatograms of the formaldehyde-containing solution revealed that IV was not formed to any significant degree confirming that the equilibrium of the reaction III → IV does not favour formation of IV. It was noted that addition of formaldehyde to ammonium-containing solutions resulted in a drop in pH from 7.8 to 5.9 due to reaction between the formaldehyde and ammonia. As the stability of ethacrynic acid in solution is known to be pH dependent [1] an additional solution was prepared and adjusted to pH 7.5 with ammonium hydroxide. Initially the rate of loss of ethacrynic acid was slower in the pH 5.9 formaldehyde-containing solution than in the formaldehyde-free solution. This effect can be attributed to the probable absence of the ammonium ion following reaction with formaldehyde and to the lower pH of the system. After the initial period, greater loss of

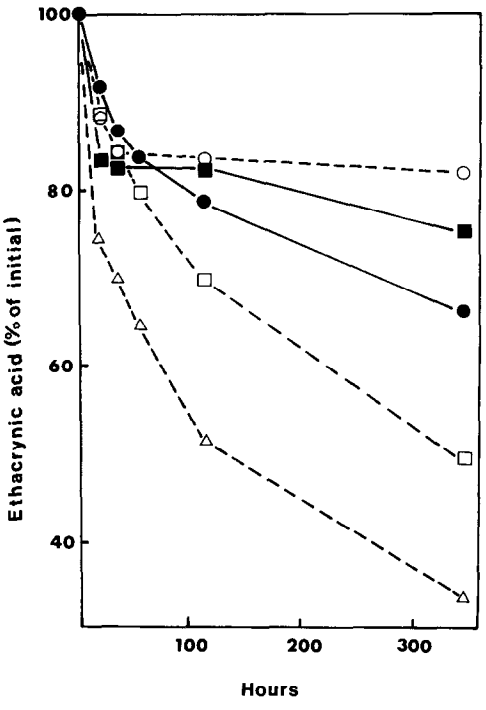


Figure 5
Influence of formaldehyde on the stability of ethacrynic acid (0.5 mg/ml) in selected buffer solutions, stored at 60°C. Key: ○, Disodium hydrogen phosphate 0.04 M, formaldehyde 0.1 M, pH 8.2; ●, Disodium hydrogen phosphate 0.04 M, pH 8.2; □, Diammonium hydrogen phosphate 0.04 M, formaldehyde 0.1 M, pH 5.9; ■, Diammonium hydrogen phosphate 0.04 M, pH 7.8; △, Diammonium hydrogen phosphate 0.04 M, formaldehyde 0.1 M, pH 7.5.

Table 2
The influence of hydroxyammonium chloride on the stability of ethacrynic acid (0.5 mg/ml) solutions refluxed for 1 h

Solution	Ethacrynic acid (% remaining)	Principal degradation product
Ammonium hydroxide 0.25 M	82	VII
Ammonium hydroxide 0.25 M + hydroxyammonium chloride, 2% w/v	Trace	IX
Sodium borate pH 9.5 0.25 M + hydroxyammonium chloride, 2% w/v	Trace	IX

ethacrynic acid was noted in the pH 5.9 formaldehyde-containing solution than in the formaldehyde-free solution and the chromatograms revealed an additional degradation product VIII with a retention time close to that of VII. The greatest loss of ethacrynic acid was sustained in the ammonium- and formaldehyde-containing solution adjusted to pH 7.5. Again, degradate VIII was seen. It is concluded that the addition of formaldehyde to ethacrynic acid in ammonium-containing solutions results in greater loss of ethacrynic acid than in formaldehyde-free solutions and that the increased loss is a consequence of an additional degradation pathway being operative. No attempts were made to isolate and identify VIII.

The presence of hydroxyammonium chloride in ammonium-containing solutions was shown to increase the rate of loss of ethacrynic acid markedly (Table 2). However, analysis of the solutions by HPLC revealed that this increased loss was due to generation

of one or more unidentified polar degradation products (IX) with retention time close to that of VII, rather than to the equilibrium of the reaction III → IV being in favour of IV, as Das Gupta had concluded. Degradation product(s) with chromatographic characteristics identical to IX were found also in a control solution buffered with sodium borate and containing hydroxyammonium chloride.

Acknowledgements: The authors thank Mr Derek Kennedy and Mr Paul Byway for carrying out the NMR and MS studies and express their appreciation to Barbara Cresswell and Joan Flexmore for secretarial assistance.

References

[1] R. J. Yarwood, W. D. Moore and J. H. Collett, *J. Pharm. Sci.* **74**, 220–223 (1985).
[2] R. J. Yarwood, A. J. Phillips, N. A. Dickinson and J. H. Collett, *Drug Dev. Ind. Pharm.* **9**, 35–42 (1983).
[3] V. Das Gupta, *Drug Dev. Ind. Pharm.* **6**, 869–882 (1982).
[4] *U.S. Pharmacopeia*, (20th rev.), 945–946 (1980). U.S. Pharmacopeial Convention Inc., Rockville, MD.
[5] R. Roman, *Pharmacopeial Forum* **8**, 2237–2238 (1982).

[*First received for review 19 June 1986; revised manuscript received 7 November 1986*]