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Note

Liquid chromatographic identification of clorazepate in pharmaceutical products

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Clorazepate is a relatively new benzodiazepine available in oral dosage forms as Tranxene and Azene. Tranxene is the dipotassium salt of clorazepate and Azene is the monopotassium salt. Product information[1,2] indicates that aqueous solutions of clorazepate are unstable and undergo rapid decarboxylation. The decarboxylation product and primary metabolite, N-desmethyldiazepam (nordiazepam), quickly appears in the blood stream following ingestion of clorazepate. Clorazepate in its original form is not normally detected in the blood and the pharmacological activity of clorazepate is essentially that of N-desmethyldiazepam. Detailed pharmacological data on N-desmethyldiazepam is available[3,4].

Recent reports[5,6] have indicated difficulties in the analysis of clorazepate. Clorazepate salts are highly water soluble with little or no organic solvent solubility. Aqueous solutions of clorazepate salts are basic and upon acidification the clorazepate rapidly decarboxylates to yield N-desmethyldiazepam. Extraction and analysis of the decarboxylation product would serve only as an indication of the presence of clorazepate and could not be used as positive identification. Direct mass spectral analysis[6] of clorazepate salts by solid probe yields ions at *m/e* values identical to those observed for N-desmethyldiazepam. This is attributed to a loss of CO_2 due to thermal decomposition on the solid probe. Infrared spectral data has been used to identify clorazepate salts[6]. This paper reports the results of our efforts to develop a liquid chromatographic method of analysis for clorazepate in pharmaceutical dosage forms.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a Waters Model 6000 solvent pump, Model U6K injector equipped with a 2-ml loop, a Model 440 UV detector and a Varian A-25 recorder. Ultraviolet absorption spectra were measured using a Hitachi Model 60 or a Perkin-Elmer Model 200 spectrophotometer. A Beckman Model 3500 pH meter was used to make all pH measurements.

Reagents

All reagents were of ACS reagent-grade quality and were used as purchased without further purification. HPLC grade methanol was obtained from Fisher Scientific (Atlanta, Ga., U.S.A.). Clorazepate dipotassium was obtained from Abbott Labs. (Chicago, Ill., U.S.A.) and N-desmethyldiazepam was supplied by Hoffmann-La Roche (Nutley, N.J., U.S.A.). The pH 8.04 buffer was prepared by mixing 5.0 ml of a stock solution of 9.2 g/l of NaH_2PO_4 and 95.0 ml of a stock solution of 17.86 g/l of Na_2HPO_4. All aqueous solutions were prepared in double distilled water.

Chromatographic procedures

Separation was accomplished using a 3.9 mm I.D. by 30 cm μBondapak C_{18} column (Waters Assoc., Milford, Mass., U.S.A.). The mobile phases described were made up of pH 8.04 phosphate buffer and HPLC grade methanol. Paired-ion chromatographic procedures were accomplished by adding sufficient tetrabutylammonium phosphate to the mobile phase to produce a 0.005 *M* solution.

Analysis of dosage forms

Samples of Tranxene and Azene were added to 10 ml of pH 8.04 phosphate buffer and the resulting suspension agitated for 5 min. The solution was filtered and a 3 μl sample of the solution analyzed by liquid chromatography.

RESULTS AND DISCUSSION

The results of our initial attempts at the analysis of clorazepate by liquid chromatography are shown in Fig. 1. A sample of clorazepate salt was dissolved in water and aliquots of the solution were injected into the liquid chromatograph. The mobile phase was water–methanol (3:7) adjusted to various acidic pH values. Chromatograms A and B in Fig. 1 were obtained by injecting 3 μl of the clorazepate

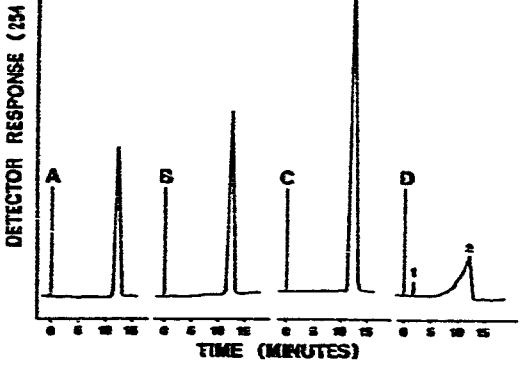

Fig. 1. Effects of mobile phase pH on clorazepate decarboxylation. Mobile phase: methanol–water (7:3) at 1.5 ml/min. Chromatograms: A, clorazepate injection at mobile phase pH 4.0; B, clorazepate injection at mobile phase pH 4.6; C, N-desmethyldiazepam injection at mobile phase pH 4.6; D, clorazepate injection at mobile phase pH 5.6 (peaks: 1 = clorazepate; 2 = N-desmethyldiazepam).

solution at a mobile phase pH of 4.0 and 4.6 respectively. Chromatogram C resulted from an injection of a known sample of N-desmethyldiazepam. As the mobile phase pH was increased to 5.6, an injection of the clorazepate solution showed two peaks (Fig. 1D). The first peak occured at a much shorter retention time than N-desmethyldiazepam and the second peak which eluted at approximately the same retention time as N-desmethyldiazepam exhibited a fronting effect. Further increases in mobile phase pH resulted in an increase in the area of peak 1 and a symmetrical shape for peak 2. This series of experiments shows that at pH levels of 4.6 and less the clorazepate sample underwent complete decarboxylation in the injector and was chromatographed as N-desmethyldiazepam. At a mobile phase pH of about 5.6 the rate of decarboxylation is slower allowing a small amount of clorazepate to elute through the octadecylsilane column to produce peak 1 and contribute to the fronting effect for the N-desmethyldiazepam peak. The fronting on the second peak is most likely produced by the decarboxylation of portions of the clorazepate sample at various points along the column. A sample of clorazepate which undergoes on-column decarboxylation would cause the elution of the resulting N-desmethyldiazepam at a shorter retention time than normal thus the fronting effect is produced. N-desmethyldiazepam was not detected in a pH 8 clorazepate solution stored at ambient temperature for 1 week. Thus, by maintaining the mobile phase at pH 8 the clorazepate decarboxylation can be prevented. Further increases in mobile phase pH must be avoided due to the dissolution of the silica based stationary phase in basic solvents of greater than pH 8[7].

The effects of pH on clorazepate salts are shown in Fig. 2. The structures shown suggest that clorazepate is at least partially ionized at pH 8 and thus ionized under the chromatographic conditions. The results of some attempts at paired-ion chromatography with clorazepate further support this conclusion. A solution of

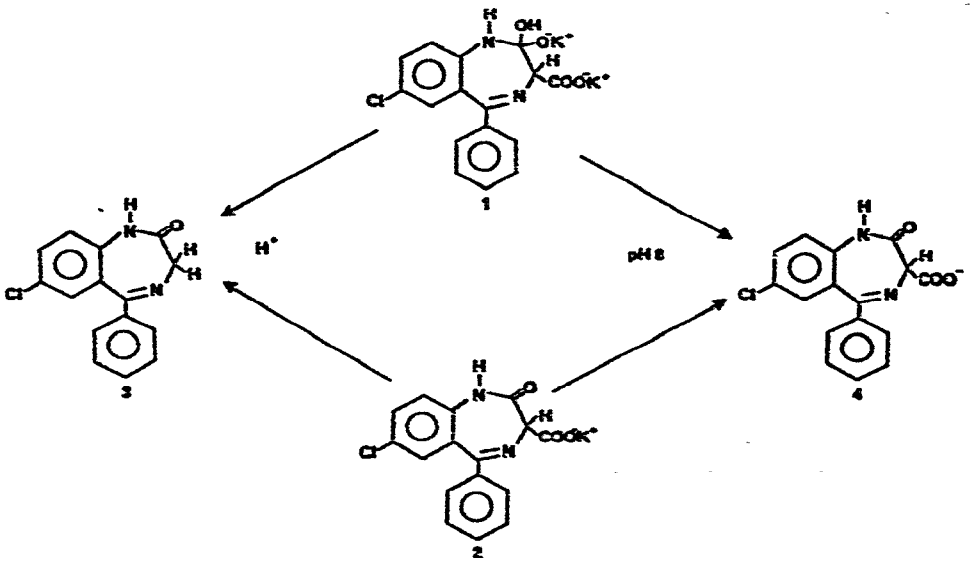

Fig. 2. The effects of pH on clorazepate stability. Structures: 1 = clorazepate dipotassium (Tranxene); 2 = clorazepate monopotassium (Azene); 3 = N-desmethyldiazepam; 4 = clorazepate anion.

clorazepate and N-desmethyldiazepam was chromatographed in methanol–pH 8 phosphate buffer (2:1) and in 0.005 *M* tetrabutylammonium phosphate prepared in the same mobile phase. The addition of the tetrabutylammonium ion produced an increase in the retention time of the clorazepate peak while the retention of the non-ionic N-desmethyldiazepam was not altered. The retention of the clorazepate–tetrabutylammonium ion-pair was only slightly greater than that observed for free clorazepate. This perhaps indicates that the octadecylsilane stationary phase has some affinity for the ionized clorazepate. The retention of organic ions by hydrocarbonaceous stationary phases has been reported in other work[8]. The mechanism of retention in reversed-phase chromatography in the presence of solvophobic ions is the topic of a great deal of current research[9]. One mechanism postulated for ion-pair chromatography suggests the retention of the organic ion-pairing agent by the stationary phase to form a reversible ion-exchange column[10]. Thus, the retention of organic ions by an octadecylsilane column is not a new concept. Further adjustments showed that an increase in the water content of the mobile phase produced the desired increase in retention for clorazepate. Due to the hydrophobic nature of the clorazepate anion the ion-pairing was not necessary for the adjustment of chromatographic conditions.

The separation of clorazepate from other commonly encountered benzodiazepines was accomplished using the octadecylsilane column and a methanol–pH 8 phosphate buffer (4:3) mobile phase. The separation is shown in Fig. 3. Dosage forms of Tranxene and Azene when dissolved in pH 8 buffer and injected into the

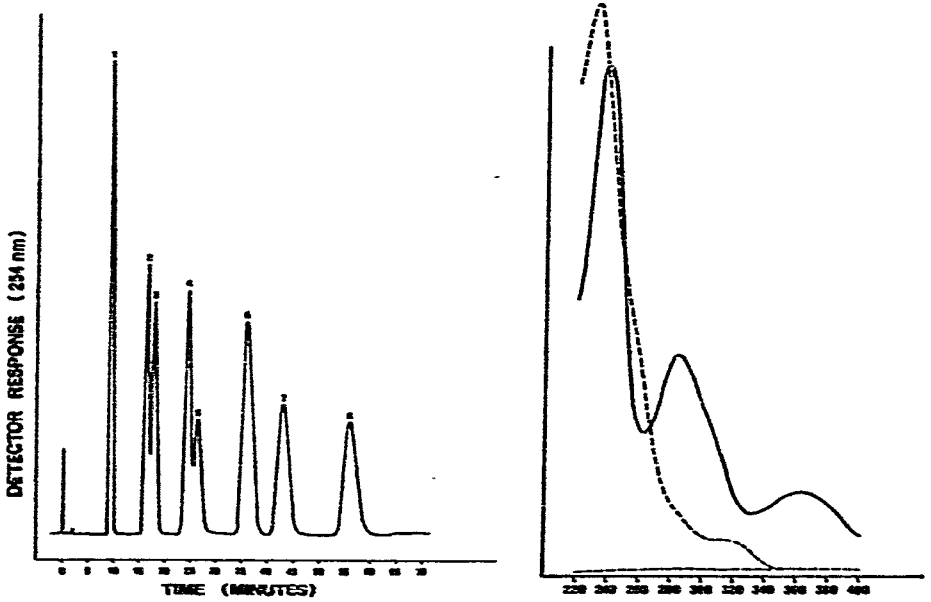

Fig. 3. Liquid chromatographic separation of benzodiazepines. Mobile phase: methanol–pH 8 phosphate buffer (4:3) at 1.5 ml/min. Peaks: 1 = clorazepate; 2 = nitrazepam; 3 = clonazepam; 4 = oxazepam; 5 = lorazepam; 6 = chlordiazepoxide; 7 = N-desmethyldiazepam; 8 = diazepam.

Fig. 4. Ultraviolet absorption spectrum of the clorazepate peak collected from the liquid chromatographic effluent. Dotted line is the spectrum obtained in the mobile phase at pH 8; solid line represents the spectrum obtained upon acidification of the mobile phase solvent system with 0.5 *N* H_2SO_4.

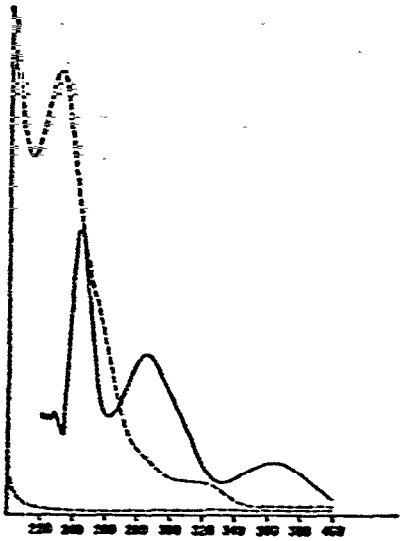

Fig. 5. Ultraviolet absorption spectrum of clorazepate standard in the liquid chromatographic mobile phase solvent system. Dotted line is the spectrum obtained at pH 8; solid line is the spectrum obtained upon acidification with 0.5 *N* H_2SO_4.

liquid chromatograph under the described conditions showed identical retention times. Thus, the two salt forms produce equivalent species in solution at pH 8 (Fig. 2).

Further analytical data can be obtained on unknown samples of clorazepate following liquid chromatographic analysis. Fig. 4 shows the UV spectrum obtained by collection of the clorazepate peak from the liquid chromatographic effluent. The spectrum was obtained first in basic solution followed by acidification of the solution which resulted in the production of the UV spectrum of N-desmethyldiazepam. Fig. 5 shows the UV spectrum for a standard sample of clorazepate obtained in basic and acidic mobile phase solvent. These spectra are consistent with UV spectra determined in aqueous acid and base[11]. Thus, clorazepate can be analyzed in its original form by liquid chromatography followed by peak collection for positive identification by UV analysis and other confirming tests. The procedure described in this paper can be used for the positive identification of clorazepate in its original form and the method is applicable to a large group of benzodiazepines.

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