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Note

An ultramicro high-performance liquid chromatographic method for assaying ion-pair species of benactyzine

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Current methodology for measuring the concentration of benactyzine in various types of therapeutic drugs is based primarily on colorimetric and spectrophotometric methods[1–4]. In most cases, pre-treatment of the sample is required prior to analysis. Hence, many of the procedures used in these methods are long and tedious. The sensitivity is limited. Benactyzine levels of less than 5 μg are difficult to measure.

We have recently developed a highly sensitive ion-pair high-performance liquid chromatographic (HPLC) method for determining benactyzine in various therapeutic drugs using reversed-phase chromatography. The method is capable of quantifying levels of the compound as low as 200 ng on column. Analysis time is 6 min per sample. No pre-treatment is required. As a rather simple and specific method, a high degree of precision and accuracy is possible when utilizing this new procedure.

EXPERIMENTAL*

Apparatus

A Waters Assoc. (Milford, Mass., U.S.A.) Model ALC/GPC 204 liquid chromatograph, equipped with two Model 6000A high-pressure pumps, a 660 solvent programmer, a U6K loop injector, a 254-nm UV detector, a Houston Instrument series A 5000 Omni-Scribe dual-pen recorder and a Columbia Scientific Supergrator-3 integrator were used to complete this study.

Reagents

All solvents used for these chromatographic separations were of spectro quality or analytical grade. Acetonitrile was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.). PIC B-7 reagent (1-heptane sulfonic acid) was purchased from Waters Assoc. Stock standard solutions of benactyzine hydrochloride, benzilic acid and *p*-sulfanilic acid (Aldrich, Milwaukee, Wis., U.S.A.) were used to prepare all working standards. Quality control was maintained by sulfanilic acid internal standards.

* The manufacturers' names and products are given as scientific information only and do not constitute an endorsement by the United States Government.

Procedure

A pre-packed 30 cm × 3.9 mm I.D. μBondapak C_{18} column (Waters Assoc.) was used to separate benactyzine and benzilic acid. The mobile phase consisted of 0.01 *M* 1-heptanesulfonic acid mixed with acetonitrile and was prepared by dissolving 20 ml of the pre-packaged reagent PIC B-7 in 480 ml of glass-distilled water. The pH of the solution was 3.40.

Utilizing both pumps of the HPLC system along with the 660 solvent programmer, a 35–65% mixture of acetonitrile to PIC reagent was isocratically pumped through the column. The flow-rate was 1.5 ml/min. Operational pressures ranged between 1200 and 1500 P.s.i. All separations were performed at ambient temperatures. Two microlitres of sample were introduced onto the column through a continuous-flow loop injector. The detection limit of the method was 100 ng on-column at the lower absorbance range of 0.005 A. Peak areas were measured by an on-line computing integrator.

RESULTS AND DISCUSSION

The recent introduction of ion-pair HPLC as a new and innovating technique has created the impetus for developing simpler[1], speedier and more sensitive analytical methods. As such, we have developed an ultramicro HPLC method to measure benactyzine in various types of therapeutic drugs. Benactyzine hydrochloride, which is a mild anti-depressant and anti-cholinergic agent is often administered to patients for reducing autonomic responses due to emotional provoking stresses.

Our primary interest for developing an improved method for quantifying benactyzine was due to the instability of this compound under certain conditions. We wanted to observe its fate when subjected to various pH and temperature gradients. Benzilic acid, the oxidative by-product, which is formed during hydrolysis, is ineffective as a therapeutic modality. This method is also capable of separating and quantifying benzilic acid.

A series of standards and experimental sample were analyzed. Fig. 1 represents the calibration curve of benactyzine standards containing concentrations ranging from 200 through 1000 ng. Each point plotted on the graph is an average of five separations for the denoted quantity. A linear relationship was observed for all absorbance

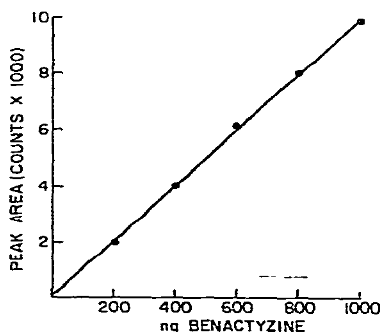

Fig. 1. Calibration curve of benactyzine detected at 254 nm, 0.005 A. Column: 30 cm × 3.9 mm I.D., μBondapak C_{18}.

ranges studied (0.005–0.02 A). The precision of the method was excellent. The coefficient of variation for these multiple analyses ranged from 0.5 to 1.2%.

In three chromatograms, which depict the separation of benactyzine and benzilic acid, the sensitivity and resolution of the method are demonstrated. Fig. 2 shows the separation of a 2-µl aqueous standard solution containing 400 ng/µl ofbenactyzine and 5 ng/µl of sulfanilic acid. This standard solution was stable for three weeks when refrigerated at 4°.

On the contrary, benactyzine hydrochloride prepared in 0.01 *N* NaOH was highly unstable. The chromatogram shown in Fig. 3 is a partially hydrolyzed sample of benactyzine, which was heated in boiling water at 90° for 30 sec. During the period

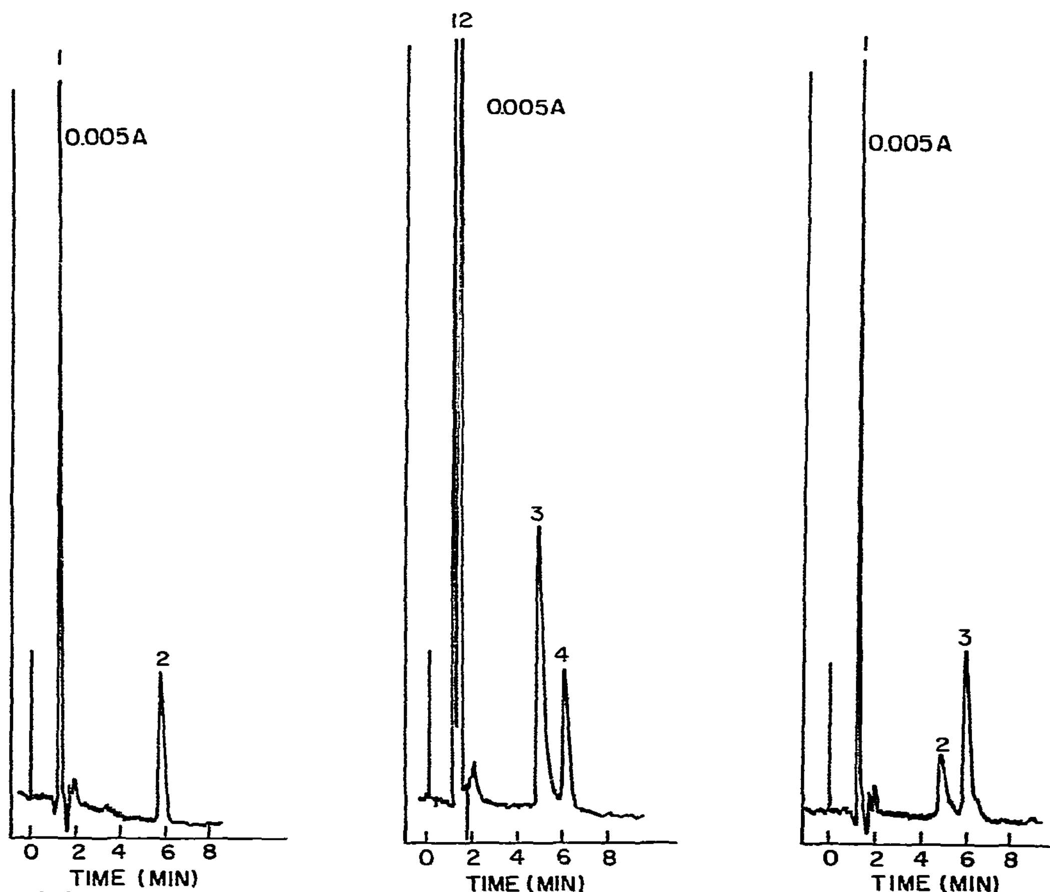

Fig. 2. Separation of a standard solution of (1) 10 ng sulfanilic acid (internal standard) and (2) 800 ng benactyzine. Mobile phase: 35% acetonitrile and 65% PIC B-7 reagent. Flow-rate: 1.5 ml/min. Column temp.: 20°. Chart speed: 0.5 cm/min.

Fig. 3. Chromatogram of a 2.0-µg sample of benactyzine, heated in 0.01 *M* NaOH at 90° for 30 sec. Peaks: (1) unknown; (2) sulfanilic acid; (3) benzilic acid; (4) benactyzine.

Fig. 4. Chromatogram showing the formation of benzilic acid in 0.01 *M* NaOH at room temperature. Separation includes (1) sulfanilic acid, (2) benzilic acid and (3) benactyzine.

of hydrolysis, 60% of the benactyzine was converted to benzilic acid. The total conversion occurred after 60 sec.

When an identical sample of benactyzine was prepared and allowed to stand at room temperature, the results were similar. Benactyzine oxidized to benzilic acid but at a much slower rate. In the chromatogram of Fig. 4, 3% of benactyzine was oxidized to benzilic acid in 10 min. Upon leaving the remaining portion of the sample overnight at room temperature, more than 90% of benactyzine had oxidized.

Conversely, benactyzine hydrochloride was relatively stable in 0.01 *N* HCl at room temperatures, 37° and 90° under similar experimental conditions as were used in the 0.01 *N* NaOH study.

From this study, we were able to demonstrate the applicability of this new analytical technique in measuring the concentration of benactyzine. Obviously, an analytical system offering the advantages of improved sensitivity, specificity and simplicity should have an immediate application in minimizing many of the disadvantages that beset conventional analytical methodologies.

The advantages of ion-pair HPLC are self-evident as was demonstrated by this study.

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