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Note

Assay for the determination of the tetracyclic antidepressant compound aptazapine in plasma by high-performance liquid chromatography

JEANNETTE DEHAAS VERMEULEN* and THOMAS A. THOMPSON

Research Department, Pharmaceuticals Division, Ciba-Geigy Corporation, Ardsley, NY 10502 (U.S.A.)

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Aptazapine {1,3,4,14*b*-tetrahydro-2-methyl-10H-pyrazino[1,2-*a*]pyrrolo-[2,1-*c*][1,4]benzodiazepine maleate} (MW 369.4) is a potential antidepressant drug [1], which is presently being evaluated in clinical studies. This tetracyclic compound is structurally related to mianserin, another antidepressant agent, which is used as the internal standard in the described assay (Fig. 1). Both compounds are basic, contain tertiary nitrogen and are naturally fluorescent. Plasma concentrations of aptazapine were expected to be at the low nanomole per liter level. Fluorometric detection was used to obtain high sensitivity. This paper gives a detailed description of a high-performance liquid chromatographic (HPLC) method for the assay of aptazapine in plasma.

MATERIALS AND METHODS

Solvents

Acetonitrile, methanol and *n*-hexane of HPLC grade were purchased from Fisher Scientific (Springfield, NJ, U.S.A.). *n*-Butylamine (96%) was obtained from Aldrich (Metuchen, NJ, U.S.A.).

Standard solutions

Aptazapine and the internal standard mianserin were obtained from Ciba-Geigy Corporation (Summit, NJ, U.S.A.). A 20 mg per 10 ml stock solution of aptazapine in acetonitrile—methanol (96:4) was prepared and dilutions in this solvent were made as needed. Mianserin (5 mg per 10 ml) was dissolved in the same solvent and then diluted hundred-fold to prepare 5 ng/μl working solutions.

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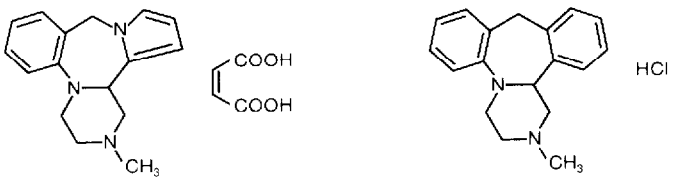

Aptazapine Mianserin

Fig. 1. Aptazapine {1,3,4,14*b*-tetrahydro-2-methyl-10H-pyrazino[1,2-*a*]pyrrolo[2,1-*c*]-[1,4]benzodiazepine maleate} and the internal standard, mianserin.

Mobile phase

The mobile phase was prepared daily and consisted of 960 ml acetonitrile, 40 ml methanol and 100 μl *n*-butylamine. The mixture was degassed by filtration through a Millipore type FH filter.

HPLC instrumentation

The chromatographic system consisted of a Waters Assoc. Intelligent Sample Processor (WISP), a Waters Model 6000A pump delivering 2.0 ml/min (Waters Assoc., Milford, MA, U.S.A.), and a Hewlett-Packard Model 3375A integrator/recorder (Hewlett-Packard, Paramus, NJ, U.S.A.). A Schoeffel fluorescence detector (Kratos, Westwood, NJ, U.S.A.) was used with the excitation wavelength fixed at 238 nm and the emitted radiation passed through a filter with a 379-nm cut-off. A Porasil guard column was fitted in front of a Hibar II silica column, particle size 10 μm, 25 cm × 4.6 mm (Merck, Cincinnati, OH, U.S.A.).

Glassware preparation

All glassware was cleaned with detergent (Micro, International Products, Trenton, NJ, U.S.A.), rinsed with distilled water and heat-treated at 800°C in an oven for 4 h. Just before use, the tubes intended for evaporations were half filled with 1% *n*-butylamine in *n*-hexane, capped and placed on an automatic shaker for 15 min, and then drained and air-dried (deactivated tubes).

Recovery measurement

[^{14}C]Aptazapine with a specific activity of 7.5 μCi/mg (Ciba-Geigy Corporation, Ardsley, NY, U.S.A.) was used to make a 1 mg per 10 ml solution in methanol, and then diluted to 4 ng/μl. Duplicate human control plasma aliquots (1.0 ml) were spiked with 10 μl of radioactive solution. The plasma was diluted with distilled water (8 ml) and 10 ml of *n*-hexane were added. The tubes were rotated for 15 min and centrifuged for 10 min. Aliquots of 9 ml of the organic extracts were transferred to scintillation vials and taken to dryness under a gentle stream of nitrogen in a waterbath at 50°C. The residues were dissolved in 100-μl aliquots of methanol with vortexing for 15 sec, after which 10 ml of Scintisol (Isolab, Akron, OH, U.S.A.) were added for measurement of radioactivity in an Intertechnique liquid scintillation counter Model SL 4000 (Fairfield, NJ, U.S.A.).

Assay procedure

Plasma (0.5—2.0 ml) was pipetted into 40-ml screwcap centrifuge tubes and 5 μl of working standard solution of mianserin (25 ng) was added as internal standard. Calibration standards ranging from 2.5 ng/ml (2.5 ng/ml÷0.369 =6.8 n*M*) to 30 ng/ml were prepared during each analysis day by adding aptazapine to control plasma aliquots to cover the range of expected concentrations for the unknowns. Distilled water (8 ml) and *n*-hexane (10 ml) were added and the tubes closed with PTFE-lined caps. Samples and standards were extracted on a rotator for 15 min and then centrifuged at 1500 *g* for 10 min. Aliquots of 9 ml of the organic extracts were transferred into deactivated tubes and evaporated under a gentle stream of nitrogen in a 50°C waterbath. Just before dryness, the inside of each tube was washed down with 1—2 ml of *n*-hexane which was then taken to dryness. All residues were dissolved in 100 μl of mobile phase with vortexing for 5 sec. The resulting solutions were transferred into Waters WISP vials and 80 μl were injected for chromatography.

Calculations

Concentrations of aptazapine were calculated from peak height ratios of drug to internal standard. Linear regression analysis was carried out with data for the calibration standards, and the resulting slope was used to obtain concentration values for the unknown samples.

RESULTS AND DISCUSSION

Mianserin has been reported to be solvent extractable in the presence of ammonium hydroxide [2]. Extraction of [^{14}C]aptazapine from plasma into *n*-hexane without changing the pH was 80%. In the extraction of aptazapine, addition of base caused endogenous interfering material to be extracted, and it did not increase recovery. Other solvents, such as toluene and chloroform were also tried as extractants, but *n*-hexane with its low polarity gave the cleanest chromatograms from control plasma extracts and was therefore chosen as the extractant for the assay.

Adsorption of drugs and internal standard onto glass, which apparently occurred during evaporation of extracts, caused variation of peak sizes in the nanogram per milliliter concentration range. Reproducibility was not improved by silanizing glassware or prerinsing with diluted hydrochloric acid. Addition of 1% isoamyl alcohol to the extractant resulted in an interfering peak in the chromatogram.

Reported methods for prevention of glass adsorption, such as circumventing the evaporation step by re-extracting into a small amount of acid [3], or wetting the glassware with aqueous alkali [4], could not be applied. In the present assay, chromatography was carried out on silica gel and this packing would not withstand the extreme acid or base conditions. The objective was to find an agent that would deactivate sites on the glassware that caused adsorption of basic amines without interfering with the chromatogram. Treatment of the glass tubes used in the evaporation step with 1% *n*-butylamine in *n*-hexane resulted in excellent reproducibility.

Difficulties with glassware contamination were avoided by using disposable

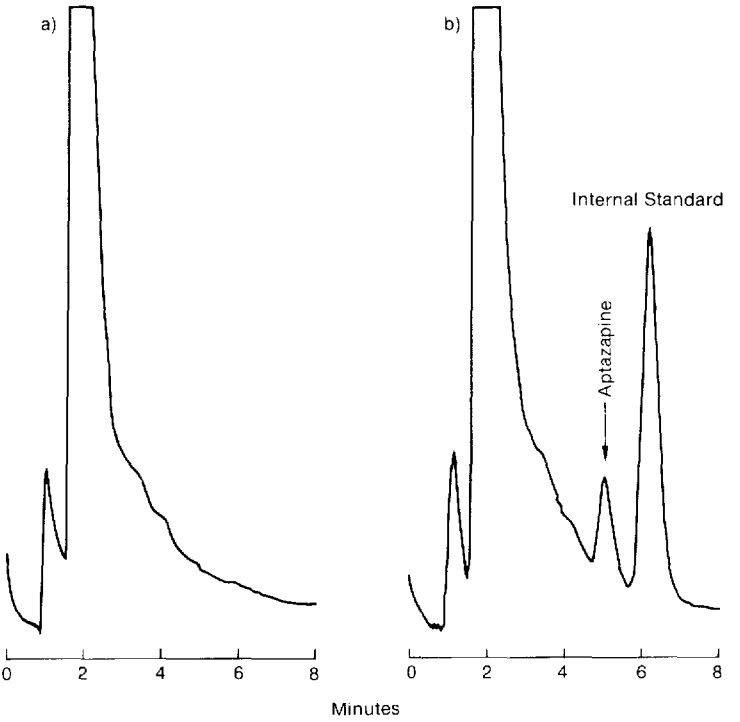

Fig. 2. Chromatograms from 2.0-ml plasma extracts: (a) plasma blank; (b) containing 2.5 ng aptazapine per ml and 25 ng internal standard.

pipets and by routinely heating all other glassware at 800°C for 4 h. This process eliminated the occasional appearance of interfering peaks.

Initial efforts at gas chromatography with a nitrogen detector were abandoned because of poor sensitivity. Reversed-phase HPLC of unextracted drug under a number of conditions produced symmetrical peaks with acceptable retention times. However, plasma extracts contained endogenous materials that interfered with the chromatograms. This interference was not resolved by modifying the chromatographic system. Pre-extraction resulted in improved chromatograms but decreased the recovery of drug. Chromatography on silica HPLC columns with a mildly polar eluent gave rather clean chromatograms but drug and internal standard peaks had long retention times accompanied by tailing. Incorporation of 1 m*M* (100 μl/l) of *n*-butylamine in the mobile phase caused elution of the drug peak, with a much improved shape, immediately after the initial plasma material. The internal standard was well separated (Fig. 2).

Eluents containing aliphatic amines or ammonium hydroxide have been used previously in HPLC separations of tricyclic antidepressants in order to decrease peak tailing and retention time [5—8]. The nature of the mechanism is not clear, although the bases may function as silica surface modifiers as suggested for water [9] and methanol [10]. The modifier presumably masks some of the more active sites on the silica by Van der Waals interaction with neutral molecules, or by acid—base interaction with the amines. The present system represents liquid—solid chromatography in normal phase mode, be-

cause no immiscible or nearly saturated component is present to form a stationary liquid phase.

It has been reported that column efficiency and column life are decreased by exposure to mobile phase of pH 8 or above and that long intervals of baseline stabilization are needed between injections [3]. These problems were not apparent in the present assay, possibly because of the low concentration of *n*-butylamine, and injections could be made every 7—8 min. Retention times decreased slightly over the course of a day's run, but flushing the column with methanol and leaving it in acetonitrile overnight resulted in retention times of the original value at the start of the next day.

The fluorometric response to increasing amounts of aptazapine injected on the column was found to be both linear and precise over the examined concentration range 10—100 ng, as indicated by a correlation coefficient of 0.9998.

The reproducibility of the assay was examined by the analysis of nine replicate aliquots of human control plasma containing 25 ng each of drug and internal standard. The mean peak height ratio of drug to internal standard had a coefficient of variation (C.V.) of 3%, indicating excellent precision.

Standard curves covering the range 2.5—30 ng/ml were obtained on five different occasions as a test of linearity and precision. The mean slope was 0.0076, with a C.V. of 4.7%. Another set of spiked samples was analyzed on each of the five occasions and concentrations were calculated as though unknown. The mean and C.V. for percent recovery were 99.7 and 8.2%, demonstrating excellent assay characteristics. The C.V. for 2.5 ng/ml samples was 7.5%.

The utility of this assay has subsequently been demonstrated by the analysis of aptazapine in plasma samples from toxicological and clinical studies. In one such study, five human subjects received 40 mg aptazapine every 12 h for five days. The mean and standard deviation for plasma concentrations were found to be 16.6 ± 11.6 ng/ml at 3 h after the last dose and 3.25 ± 0.99 ng/ml at 12 h. Concentrations as low as 2.5 ng/ml plasma have been measured and 1.25 ng/ml is measurable if the plasma aliquot is doubled.

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