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Note

Rapid method for determination of ketanserin, a novel antiserotonin drug, by high-performance liquid chromatography

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Ketanserin (R-41,468) is a novel synthetic drug (Fig. 1) which has been reported to be a highly selective antagonist of the peripheral serotonergic$_2$

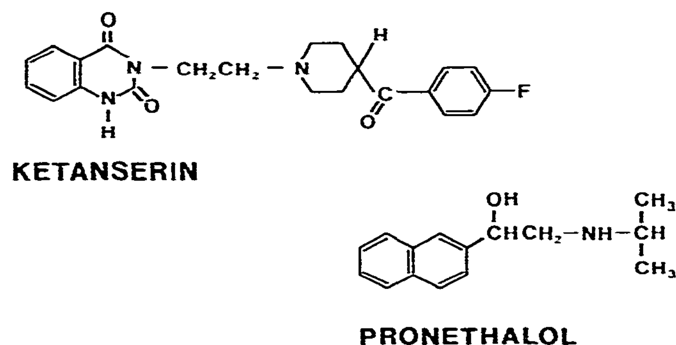

Fig. 1. Chemical structures of ketanserin and the internal standard, pronethalol.

receptors [1]. In the isolated preparations of either arterial or venous vascular segments, ketanserin competitively blocked the direct vasoconstrictor effects of serotonin (5-hydroxytryptamine) thus suggesting its antagonist effect on both resistance and capacitance blood vessels. This drug also caused a dose-dependent reduction in arterial blood pressure of spontaneously hypertensive rats. The exact role of serotonin in the pathophysiology of hypertension, congestive heart failure or other cardiovascular diseases is presently not known, but recent experimental data suggest that serotonin potentiates the vasoconstrictor response of norepinephrine on the alpha adrenergic receptors [1] and it may play some role in these diseases. Ketanserin also inhibits this

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potentiating effect of serotonin$_2$ receptors since it does not inhibit the response mediated via the adrenergic, cholinergic or other receptors [1]. Ketanserin is currently being investigated for clinical management of hypertension [2] and congestive heart failure [3]. We have developed a simple and rapid method for measurement of ketanserin in human plasma using reversed-phase high-performance liquid chromatography (HPLC) in order to enable us to correlate pharmacodynamic response with plasma drug levels in patients receiving this drug.

EXPERIMENTAL

Chemicals and reagents

Ketanserin, {3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl] ethyl] -2,4(1H,3H)-quinazoline dione} was provided by Janssen Pharmaceutical (New Brunswick, NJ, U.S.A.). The internal standard, pronethalol {α-[isopropylamino)-methyl] -2-naphthalenemethanol}, selected because its chemical structure suggested the possibility of similar extraction and identification characteristics by the HPLC techniques, was obtained from Ayerst Labs. (New York, NY, U.S.A.). Pronethalol is not used clinically and therefore no interference would be expected in patients who may receive ketanserin. HPLC-grade acetonitrile was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Methylene chloride was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All other chemicals were reagent grade.

Instrumentation

All HPLC components were manufactured by Waters Assoc. (Milford, MA, U.S.A.) and included: 6000A solvent delivery system, U6K injector, and Model 440 UV absorbance detector with the wavelength fixed at 254 nm. The recorder was a Houston OmniScribe with a 10-mV output.

Chromatographic conditions

The column was a Waters μBondapak phenyl reversed-phase, 30 cm × 3.9 mm I.D., with particle size of 10 μm. The flow-rate was set at 1 ml/min which produced a precolumn pressure of 69.0 bar. Detection was at 254 nm with sensitivity at 0.02. The chart speed was 0.5 cm/min.

Mobile phase preparation

For preparation of 2 l of mobile phase, 5.44 g of potassium dihydrogen phosphate was dissolved in ca. 1200 ml of water and 608 ml of acetonitrile was added. The pH was adjusted to 2.3 by adding approximately 19 ml of 42% phosphoric acid. The resulting solution was diluted with water to 2 l in a volumetric flask. A type FH 0.5-μm Millipore filter was wetted with acetonitrile, and the mobile phase was filtered, discarding the first small portion.

Extraction procedure

Plasma (1 ml) was added to glass tubes with PTFE-lined screw caps followed by addition of pronethalol hydrochloride (1 μg) in aqueous solution. After vortexing, a solution containing 2 *M* sodium hydroxide and 4 *M* sodium

chloride (200 μl) was added, followed by methylene chloride (5 ml). The tubes were placed on a wheel tilted at a 45° angle and rotated for 20 min at 40 rpm. The tubes were then centrifuged at 2120 *g* (bench top centrifuge) for 10 min and the upper plasma layer was removed by aspiration. If the plasma layer extended into the organic layer, it was removed by gentle shaking and, if necessary, repeated centrifugation. The organic layer was transferred to a conical centrifuge tube and evaporated to dryness in water which was gradually warmed from 45°C to 80°C. To the cooled tubes were added 0.8 *M* phosphoric acid (150 μl) and *n*-pentane (500 μl). The tubes were vortexed vigorously for 90 sec and centrifuged at 2120 *g* for 5 min. The aqueous layer was frozen by immersion in dry ice—ethanol for 1 min and the *n*-pentane layer was removed by aspiration. The pH of the aqueous layer was adjusted to 6.6 by addition of 3 *M* sodium hydroxide (50 μl). A 100-μl aliquot of the resulting 200 μl of aqueous extract was injected into the HPLC apparatus. The steps involved in the extraction of ketanserin from the plasma are summarized in Fig. 2.

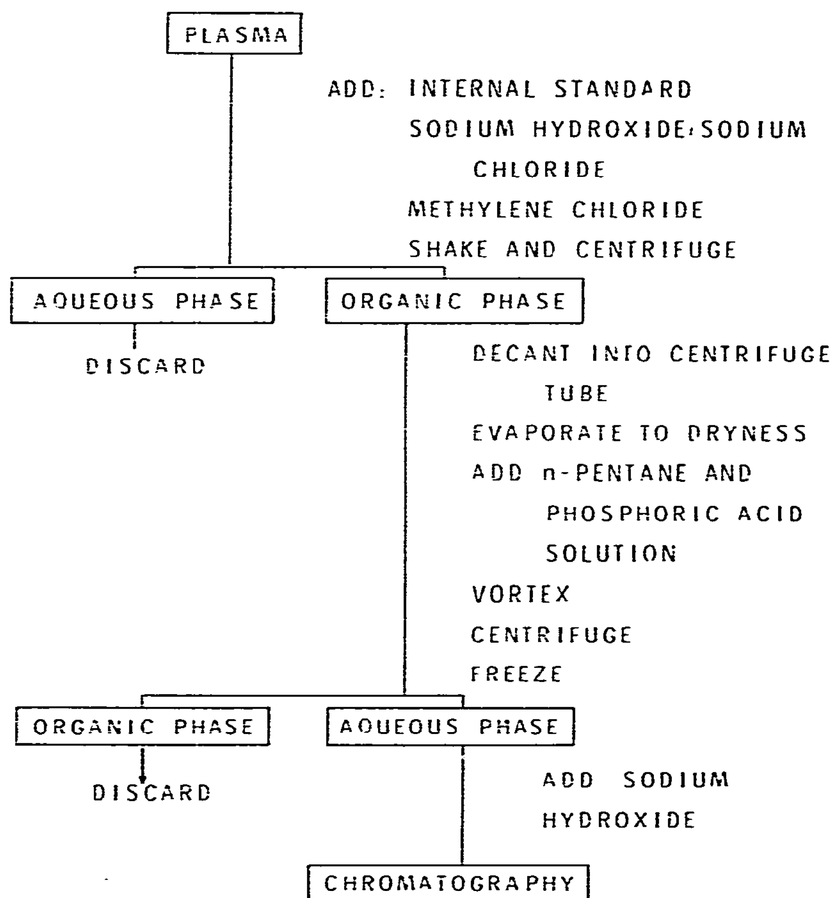

Fig. 2. Flow diagram of the procedure for the extraction of ketanserin from plasma.

Preparation of calibration standards

Ketanserin (free base) was dissolved in 0.8 *M* phosphoric acid and diluted with distilled water to produce a solution containing 2 ng/µl ketanserin and $5.8 \cdot 10^{-5}$ *M* phosphoric acid. Aqueous pronethalol·HCl at a concentration of 20 ng/µl was used as the internal standard. Although fresh solutions of ketanserin and pronethalol were prepared daily to generate the data presented in this report, both solutions appear to be stable for at least one month when stored at 4°C.

RESULTS AND DISCUSSION

In the procedure described above, the retention times for pronethalol and ketanserin on this column were found to be 7.0 and 9.8 min, respectively. The chromatogram of a plasma sample obtained from a patient 0.5 h following the intravenous injection of 10 mg of ketanserin is shown in Fig. 3. This

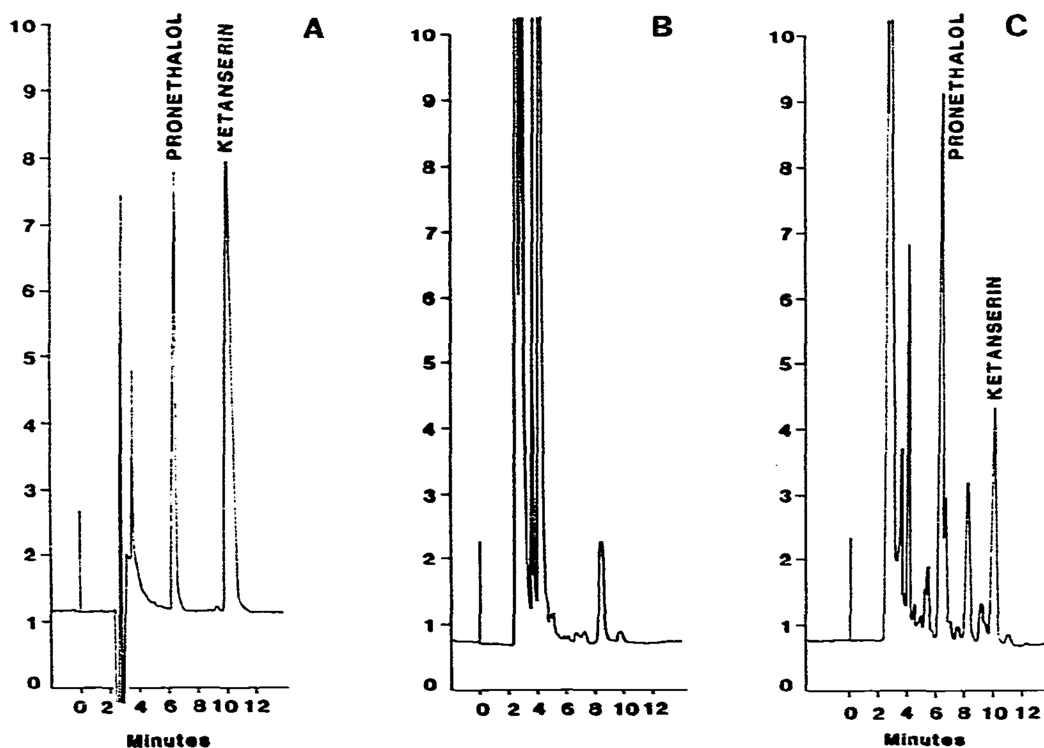

Fig. 3. High-performance liquid chromatograms of ketanserin and pronethalol (internal standard) extracted from a plasma sample obtained from a patient. Blood was drawn before and at 0.5 h after intravenous infusion of 10 mg ketanserin. In each trace, the absorbance is shown along the vertical axis and the retention time along the horizontal axis. (A) Aqueous solution of pronethalol and ketanserin; (B) control plasma and (C) post-ketanserin plasma of a patient who was given 10 mg of the drug intravenously. The peaks for ketanserin and pronethalol can be easily distinguished from other unidentified peaks in this patient (see blank, B). The calculated concentration of ketanserin in this patient was found to be 0.235 mg/l.

patient was a 45-year old white male suffering from congestive heart failure secondary to cardiomyopathy of unknown etiology. He also was concurrently receiving digoxin, furosemide, valium and acetaminophen. It may be seen (Fig. 3) that both pronethalol and ketanserin peaks were easily identified in the plasma of this patient and these peaks correlated well with those obtained with an aqueous solution containing ketanserin and pronethalol. It should be pointed out that the retention times of both ketanserin and pronethalol were found to increase if either the pH or the water content of the mobile phase was increased.

The standard curve for ketanserin was obtained in a series of experiments in which varying amounts of ketanserin ranging from 0.05 to 0.25 μg/ml and a fixed concentration of the internal standard (1 μg/ml pronethalol) were added to the plasma obtained from normal human volunteers. After the extraction procedure described above, the samples were injected into the HPLC apparatus, the peak heights of both ketanserin and pronethalol were carefully determined, and the peak height ratio, H_k/H_p, was calculated for each concentration. Linear regression of the drug concentration versus peak height ratio showed an excellent fit ($r^2 = 0.98$) and yielded the equation:

$$[K] = (0.5046 \pm 0.0053)\, H_k/H_p \ (n = 24) \tag{1}$$

where $[K]$ = calculated concentration of ketanserin free base (μg/ml), H_k = height of the ketanserin peak and H_p = height of the pronethalol peak. The coefficient of the peak height ratio in eqn. 1 was based on the addition of a fixed amount (1 μg/ml) of pronethalol to the plasma samples. However, it may be pointed out that this coefficient is proportional to the quantity of pronethalol added, and therefore if a different amount of the internal standard were used, a new coefficient should be derived.

The calculated values of ketanserin ($[K]_{calcd.}$) from the measured peak height ratio shown in eqn. 1 were correlated with the actual ketanserin con-

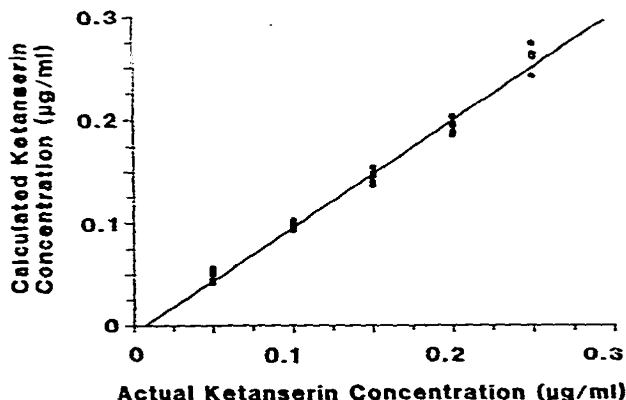

Fig. 4. Linear regression analysis of the actual ketanserin concentration added to the plasma (horizontal axis) versus calculated concentration from the peak height ratios (vertical axis). Each point represents one sample. The calculated regression line showed an excellent fit with $r^2 = 0.99$.

centration ($[K]_{actual}$) added to the plasma by the linear regression analysis (Fig. 4) and the following equation was obtained:

$$[K]_{calcd.} = 0.007 \pm 1.036\ [K]_{actual} \quad (2)$$

Data obtained by this technique showed an excellent correlation of the measured and actual ketanserin concentration ($r^2 = 0.99$).

Reproducibility of the results obtained by this technique was evaluated by replicate analysis of five plasma samples containing ketanserin at each of the following five concentrations: 0.05, 0.1, 0.15, 0.2 and 0.25 μg/ml. The amount of calculated vs. actual plasma concentration was again found to show an excellent correlation and reproducibility, since the coefficients of variation were 9.5, 1.6, 4.3, 3.0 and 8.8%, respectively.

The extraction efficiency was determined by comparing the peak height of the entire aqueous extract of ketanserin with the peak height of the same quantity of ketanserin in the aqueous solution. The recovery of ketanserin was 82 ± 8% ($n = 25$).

The entire procedure including sample preparation, extraction and HPLC determination of ketanserin can be easily carried out by a single person and it may be possible to perform as many as 15—20 drug assays per day. In order to permit batch analysis, we routinely carry out the extraction procedure the day prior to the drug assay and we have found the results to be consistent with the above data. These results suggest that the HPLC assay for ketanserin is simple, rapid, reproducible and sensitive in the range of expected plasma concentrations in patients receiving this new drug and the method reported here should prove to be useful for determination of the clinical pharmacokinetics of ketanserin.

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