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Note

High-performance liquid chromatographic assay with fluorometric detection of ketanserin, a new antihypertensive agent and serotonin S_2 antagonist in human plasma, blood and urine

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Ketanserin (R 41468) or 3-{2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl}-2,4-(1H, 3H) quinazolinedione (see Fig. 1) is an antagonist of 5-hydroxytryptamine S_2 receptors (5-HT_2) [1, 2] and a novel antihypertensive agent undergoing current widespread clinical investigations [3–7]. To date, only limited information is available concerning the concentration range of this drug in plasma, blood or urine. A radioimmunoassay method has been developed [8], but it is not generally available and its specificity has not yet been confirmed. Evaluation of pharmacokinetic data and plasma level monitoring might contribute to a better understanding of the therapeutic efficacy and mechanisms of action of ketanserin.

We report here a sensitive and selective high-performance liquid chromatographic (HPLC) assay with fluorometric detection for ketanserin. Following its extraction from plasma, whole blood or urine, ketanserin can be measured in

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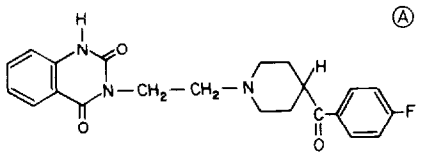
humans after therapeutic doses. Pharmacokinetic data derived from this plasma level monitoring will provide the rational basis for establishing proper dosage regimens of this new drug.

EXPERIMENTAL

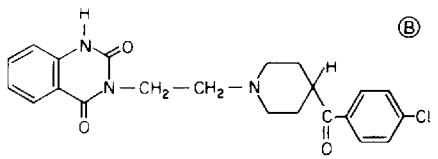
Chemicals and reagents

Ketanserin and the internal standard (R 46594) were obtained as reference compounds from Janssen Pharmaceutica, Life Sciences Products Divison, Beerse, Belgium. Chemical structures are shown in Fig. 1.

Acetonitrile, methanol, pentane and isoamyl alcohol were of nanomolar grade. Diethylamine was purified before use by elution through basic aluminium oxide, activity 60. All other reagents were of analytical grade and were purchased from Merck, Darmstadt, G.F.R. All inorganic reagents were prepared in double-distilled water.



R 41 468 (Ketanserin)



R 46594 (internal standard)

Fig. 1. Structures of ketanserin (A) and internal standard (B).

Apparatus

A Spectra-Physics solvent delivery system Model 140B was used for analysis. The pump was equipped with a Rheodyne 100-μl injection loop. For detection a Jobin Yvon Spectrofluo JY3D LC fluorimeter with excitation and emission wavelengths set at 270 nm and 410 nm, respectively, was used. The fluorometric detector was coupled to a Servocord recorder with chart speed set at either 300 mm/h or 120 mm/h. A 25 cm × 2 mm I.D. reversed-phase column equipped with a 5-cm precolumn, both packed with Nucleosil 10 μm (Macherey and Nagel, Düren, G.F.R.), were used for separation. The mobile phase was made up of water—acetonitrile (70:30) with 0.2% (w/v) diethylamine. The flow-rate was 1.00 ml/min at a pressure of 200 atm.

Sample preparation

Stock solutions of ketanserin and internal standards were stored in amber-coloured bottles at 4°C. One millilitre of quality control plasma, patient or standard plasma containing between 2 and 20 ng/ml was mixed with 0.200 ml of internal standard solution (100 ng/ml). Plasma containing high concen-

trations of ketanserin was diluted with blank plasma. Plasma or blood was alkalinized with 1 ml of 0.01 *M* sodium hydroxide in stoppered tubes; 2 ml of pentane—isoamyl alcohol (95:5) solution were added and the mixture was rotated for 10 min. Samples were centrifuged and the organic upper layer was transferred to 15-ml conical tubes. The alkalinized plasma was re-extracted with 2 ml of pentane—isomayl alcohol and the combined organic layers were evaporated under nitrogen at about 60°C. The residue was redissolved in 0.1 ml of mobile phase and injected into the HPLC column.

For urine samples buffering was needed. Accordingly, standards were prepared in 0.1 ml of blank urine and 0.01 *M* phosphate buffer (0.9 ml) pH 7.4. Aliquots (0.05—0.1 ml) of refrigerated urine were then extracted as described above for plasma.

Assignment of peaks

Qualitative designation of peaks was done by comparing their retention times to those of known reference compounds. Under our experimental conditions the retention volume of ketanserin was 4.8 ml while that of internal standard was 7.8 ml (retention times were 4.8 and 7.8 min, respectively). Ketanserin in samples was determined by calculating the ratios of peak heights of the drug to that of internal standard and relating this to concomitantly constructed calibration curves over the concentration range 2—20 ng/ml. Recovery was calculated from comparison between directly injected standards and standards subjected to the extraction procedure.

RESULTS AND DISCUSSION

Blanks from different biological samples did not show any interfering peaks even at maximum instrument sensitivity settings (Figs. 2A, 3A and 4A). A plot

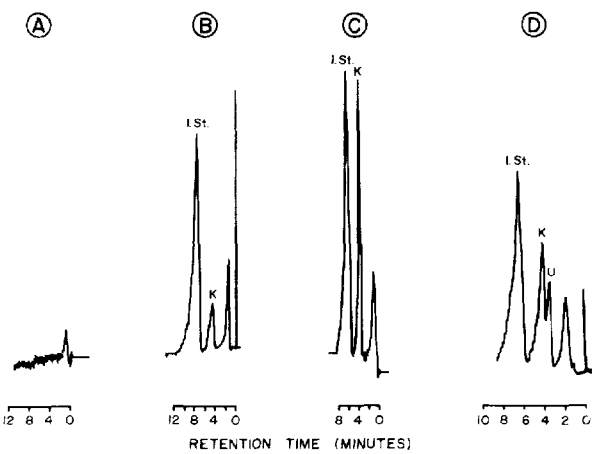


Fig. 2. Representative chromatograms of ketanserin in plasma. (A) Blank Plasma. (B) Quality control plasma: 6 ng of ketanserin (K) and 20 ng of internal standard (I. St.). (C) Standard: 20 ng of ketanserin and 20 ng of I.St. with mobile phase buffered with 0.01 *M* phosphate buffer pH 7.4. (D) A ten-fold dilution of plasma from a healthy volunteer 120 min after 40 mg oral dosing; U is a proposed metabolite. Chart speed 300 mm/h; chart speeds A, B, C were run at 120 mm/h.

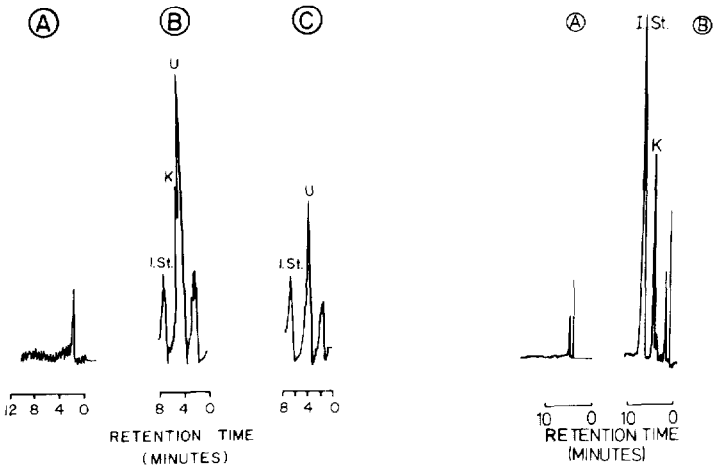


Fig. 3. Chromatogram of ketanserin in urine. (A) Blank urine. (B) A ten-fold dilution of first 24-h urine collection. (C) A ten-fold dilution of second day urine collection. I.St. = internal standard, K = ketanserin, U = unidentified metabolite.

Fig. 4. Chromatogram of ketanserin in whole blood showing (A) blank and (B) patient 1 h after an intravenous dose.

of peak height ratio versus concentration of ketanserin is linear (range 0.1—30 ng/ml) and is described by the equation $Y = 0.033X - 0.0014$ ($r = 0.9998$).

A concentration of 500 pg of ketanserin per ml plasma can be measured with reasonable accuracy. The precision of the assay was established by multiple measurements of quality control samples (12 ng/ml) stored at —20°C. The intra-assay mean ± S.D. was 12.05 ± 0.38 ng/ml and the inter-assay mean was 11.68 ± 0.36 ng/ml. The intra-and inter-assay coefficients of variation were 3.1% and 3.2%, respectively ($n = 18$). Ketanserin seemed to be stable if stored in plasma for over a three-month interval at —20°C. Frequent freezing and thawing did not reduce the reliability of ketanserin determination. Recovery was highest for plasma and lowest for urine (see Table I). At pH 7.4, fluorescence of ketanserin is enhanced over internal standard but the two compounds tended to elute too closely together (Fig. 2C). Therefore a more alkaline pH has been chosen for better separation but with some loss of sensitivity. At pH > 10.5 or < 1.5 the stability of the column was rather poor.

TABLE I

RECOVERY OF KETANSERIN IN BIOLOGICAL SAMPLES

	Recovery (% ± S.D.)	n
Plasma	94.8 ± 5.32	10
Whole blood	92.1 ± 6.81	8
Urine	78.5 ± 4.54	15

The assay seems to distinguish between ketanserin and a possible metabolite with a retention time of about 4 min. This peak is absent in ketanserin plasma solutions stored at −20°C, in ketanserin standards and blanks, but promptly appears 0.5 h after intravenous administration and 1 h after oral administration (designated "U" in Figs. 2D, 3B and 3C). This substance is also present in 24-h urine and is probably the main substance in 24-h urine of the second day of collection (see Fig. 3).

The metabolism of ketanserin by humans is at the present time poorly understood. We cannot therefore predict the rate of metabolite formation by our assay method. With the availability of purified reference standards of metabolites of ketanserin in the future, our method may be adapted for their easy estimation. In its present state the method is not suitable for the quantitation of ketanserin in urine when the concentration of the compound U is higher than that of ketanserin. We have also compared our HPLC assay with the radioimmunoassay method on spiked plasma samples and there appears to be a good correlation (see Fig. 5). The radioimmunoassay method was not suitable for determination of whole blood, and urine had to be fortified with blank plasma before being subjected to radioimmunoasay.

Fig. 6 depicts a representative ketanserin plasma concentration—time profile in a healthy volunteer following single dosing. This indicates that the assay can be used in pharmacokinetic and clinical studies.

In conclusion, the fluorometric detection of our HPLC method enables selective and very sensitive ketanserin measurements in biological samples. It is suitable for detection of very low ketanserin levels in biological fluids which will be an important prerequisite for determination of reliable pharmacokinetic data. Following the elucidation of the ketanserin metabolism in man and the availability of pure ketanserin metabolites as reference substances the method will have to be optimized for measuring ketanserin and its major metabolites.

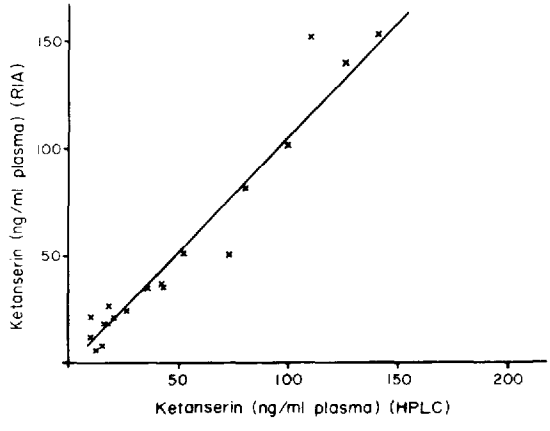


Fig. 5. Comparison of HPLC method for ketanserin with a radioimmunoassay (RIA) method. $Y = 0.998X + 0.026$, $r = 0.99$, $p < 0.001$.

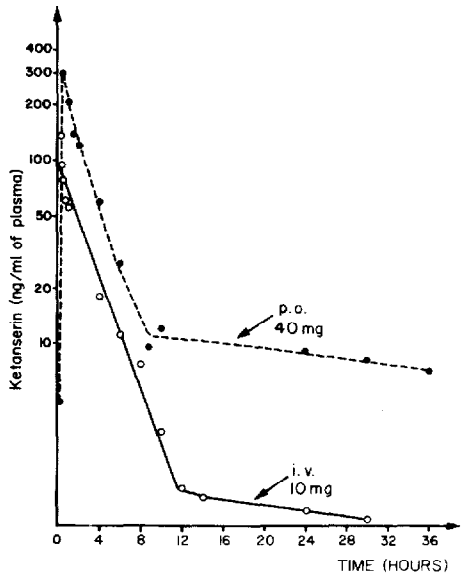


Fig. 6. Plasma concentration–time profiles of ketanserin in one subject following a single oral (p.o.) dose of 40 mg of ketanserin and a single intravenous (i.v.) dose of 10 mg of ketanserin. The pharmacokinetic parameters in this particular healthy volunteer were: oral bioavailability = 76%; elimination half-life time ($t_{½\beta}$) = 14.8 h and 11.4 h after intravenous and oral application, respectively; total body plasma clearance = 535 ml/min and 624 ml/min after intravenous and oral application, respectively; apparent volume of distribution ($V_{d\beta}$) = 10.4 l/kg and 9.3 l/kg after intravenous and oral application, respectively. Calculations were made on the basis of a two-compartment open model using a SAAM 25 computer program.

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