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Note

Determination of marplan in human plasma using high-performance liquid chromatography

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Marplan (isocarboxazid), 1-benzyl-2-(5-methyl-3-isoxazoloyl-carbonyl)-hydrazine, is a potent monoamine oxidase inhibitor [1] which has been used to treat a variety of depressive and anxiety states [2–5] as well as bulimia [6]. The drug has been shown to be extensively metabolized in vitro (rat liver homogenate) [7] and in vivo in the rat, with the majority of the dose being excreted in the urine [8].

Despite the fact that marplan has been available for clinical use for twenty years, the lack of appropriate assay methodology has resulted in no blood concentration data being generated for the drug.

In this paper we describe a quantitative high-performance liquid chromatographic (HPLC) assay for marplan in human plasma, which utilizes reversed-phase chromatography on a C_{18} column with UV detection at 230 nm. The method has also been validated for dog plasma and was used to determine marplan plasma concentrations following a single 10-mg oral dose.

EXPERIMENTAL

Chemicals and reagents

Marplan (isocarboxazid) and the internal standard (I.S., Ro 5-1226) (Fig. 1) were obtained from the Quality Control Department, Hoffmann-La Roche

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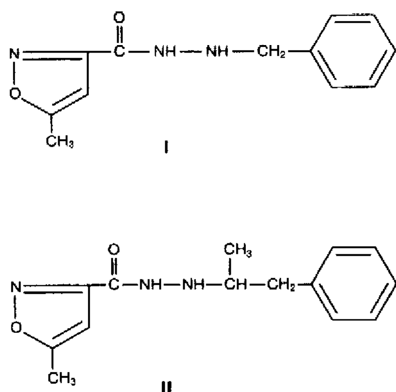

Fig. 1. Structures of marplan (I) and I.S. (II).

(Nutley, NJ, U.S.A.). Dibasic potassium phosphate and sodium hydroxide were obtained from Mallinckrodt (Paris, KY, U.S.A.). Hydrochloric acid, reagent ACS grade, methanol, hexane and ethyl acetate, all HPLC grade, were obtained from Fisher Scientific (Springfield, NJ, U.S.A.). Octanesulfonic acid–Waters Pic B-8 ion-pairing reagent (low UV) was obtained from Waters Assoc. (Milford, MA, U.S.A.). All water was distilled and deionized.

Standard solutions

Marplan solutions. Marplan (10.0 mg) was dissolved in 10 ml of HPLC-grade methanol to obtain a stock solution of 1 mg/ml marplan, which was stored at −20°C. Spiking solutions of 100, 50, 20, 10, 5 and 2 μg/ml were obtained by dilution with 0.1 *M* hydrochloric acid and were stored at 4°C.

Internal standard stock solution. I.S. (10.0 mg) was dissolved in 10 ml of HPLC-grade methanol to obtain a solution of 1 mg/ml which was stored at −20°C.

I.S. spiking solution. A 2-ml volume of the 1 mg/ml I.S. stock solution was added to a 10-ml volumetric flask and brought to volume with 0.1 *M* hydrochloric acid to give a solution of 200 μg/ml, which was stored at 4°C.

Chromatographic conditions

The HPLC system consisted of a Waters 600 multi-solvent delivery system (Waters Assoc.) with a Perkin-Elmer ISS-100 autosampler (Perkin-Elmer, Norwalk, CT, U.S.A.). The separation was performed on a prepacked 15 cm × 4.6 mm I.D. stainless-steel column containing 5-μm Supelco LC-18 packing (Supelco, Bellefonte, PA, U.S.A.). The UV detector was a Waters 481 LC spectrophotometer and was operated at a wavelength of 230 nm and a sensitivity of $1 \cdot 10^{-2}$ a.u.f.s. A Spectra-Physics SP4290 integrator (Spectra-Physics, San Jose, CA, U.S.A.) was used to record chromatograms and calculate

peak heights of marplan and I.S. The isocratic mobile phase was 0.005 *M* octanesulfonic acid–methanol (50:50, v/v) at a constant flow-rate of 1.0 ml/min for 16 min, resulting in a column pressure of 107 bar. Under these conditions, marplan eluted in 6.4 min and the I.S. in 15.5 min.

Procedure

Calibration curve samples (prepared in duplicate). To 0.5 ml of drug-free control human plasma were added 0.05, 0.125, 0.25, 0.50, 1.25 and 2.5 μg of marplan and 2 μg of I.S. in 15-ml disposable conical centrifuge tubes with screw caps.

Quality assurance samples. Samples of known concentrations were prepared at two levels, low (600 ng/ml) and high (3000 ng/ml), in order to check the accuracy of the method. Aliquots (0.5 ml) of these samples were extracted along with each standard curve and the concentrations were calculated based on linear regression analysis of the standard curve. Calculated concentrations were then compared to the theoretical concentrations to check the method.

Extraction

Samples were thawed, 1-ml aliquots of each sample were placed in tubes, 200 μl of 1.5 *M* sodium hydroxide were added to each tube along with 2 μg of I.S., and the tubes were vortex-mixed. A 5-ml volume of ethyl acetate–hexane (2:8, v/v) was added to each tube. The tubes were capped and placed on a mechanical shaker at low speed for 10 min, followed by centrifugation at 1100 *g* for 10 min at 10°C. The organic layer was transferred to a 15-ml conical tube which contained 500 μl of 2 *M* hydrochloric acid. The tubes were capped and placed on a mechanical shaker for 5 min, followed by centrifugation at 1100 *g* for 5 min at 10°C. After removing and discarding the organic phase, 200 μl of saturated dibasic potassium phosphate (K_2HPO_4) were added to the remaining aqueous layer and the sample was vortex-mixed before transferring to 1-ml autosampler vials for injection. An injection volume of 50 μl was used to analyze the samples.

Calculations

The measured peak heights were used to calculate the ratio of peak height for marplan to the peak height for I.S. at each marplan concentration. A calibration curve was obtained by plotting the peak-height ratios against the concentrations of marplan in the standards. The slope and intercept of the calibration curves were calculated using weighted ($1/y$) linear regression. Concentrations of marplan in the experimental dog samples were calculated using the equation x (ng) $= (R-b)/m$, where R is the ratio of marplan to I.S. in an experimental sample and b (intercept) and m (slope) were constants generated by the linear regression analysis of the calibration curve data.

Dog study

A single male beagle dog (10.5 kg) was administered a 10 mg/kg oral dose of marplan in a gelatin capsule. Food and water were allowed ad libitum prior to dosing and during sample collections. Blood samples were drawn into heparinized syringes through a jugular cannula. Approximately 7 ml of blood were taken pre-dose, 15, 30, 45 min and 1, 2, 3, 4, 6, 8 and 10 h after dosing. The blood samples were centrifuged for 10 min and the separated plasma was transferred to glass scintillation vials and stored at −70°C until assay.

RESULTS AND DISCUSSION

Typical chromatograms from (A) control human plasma, (B) human plasma spiked with 100 ng/ml marplan and (C) human plasma spiked with 2500 ng/ml marplan are shown in Fig. 2. I.S. was spiked at 2000 ng/ml in all samples. The retention times of marplan and I.S. were 6.3 and 15.5 min, respectively. There were no interfering peaks in any control plasma samples at either the retention time of marplan or I.S.

The linearity of the method was demonstrated by duplicate analysis of control human plasma spiked at six different concentration levels of three separate analytical runs. The mean correlation coefficient for the fit to the linear equation was 0.9993, indicating a good fit of the data to a linear model. The limit of quantitation, defined as the lowest concentration which can be accurately and reliably quantitated (relative standard deviation, R.S.D. $\leq$15%), was 100 ng/ml.

The percentage recovery of the method was determined by comparing the response of marplan recovered from spiked control human plasma to the response of marplan spiked in 0.5 ml of 2 *M* hydrochloric acid and 0.2 ml of saturated K_2HPO_4. The recovery, determined over the range 100–5000 ng/ml, ranged from 79 to 94% with an overall mean of 83%.

The inter-assay precision of the method was evaluated over a concentration range of 100–5000 ng/ml (Table I). The mean, standard deviation (S.D.) and R.S.D. were calculated based on three separate standard curves with two samples at each concentration, giving a total of six values at each concentration. The overall R.S.D. was found to be 2.6%, indicating excellent precision over this concentration range.

The intra-assay precision for the method was demonstrated by the reproducibility of duplicate determinations (one member of a set of duplicates divided by the other) of each point in the calibration curve and the quality assurance samples. The overall R.S.D. was 5.4% (Table II).

Table III contains data showing the concentration of analyte in a sample left on the bench top at room temperature (25°C) for up to 6 h and frozen at −20°C for up to fourteen days. Each result is the mean of five duplicate determinations. Samples were analyzed and compared to a 0-h sample. The re-

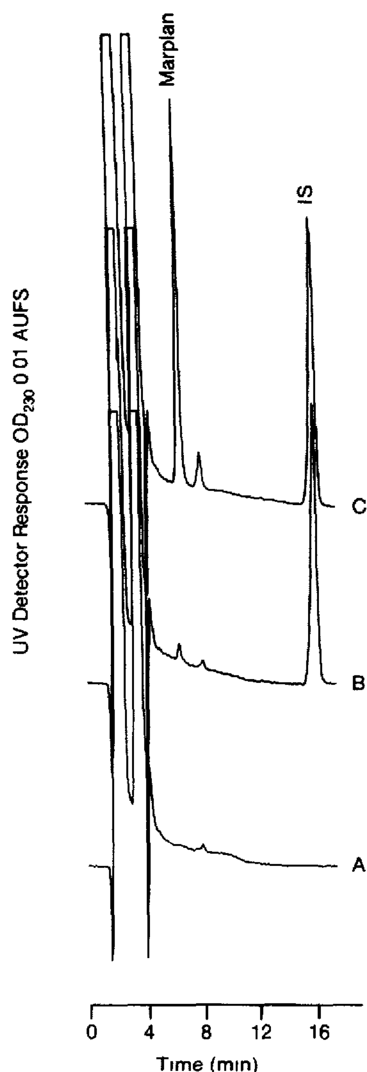

Fig. 2. Typical chromatograms from (A) control human plasma, (B) human plasma spiked with 100 ng/ml marplan and (C) human plasma spiked with 2500 ng/ml marplan. Internal standard (IS) was spiked at 2000 ng/ml in (B) and (C).

sults indicated that marplan is stable in human plasma for 6 h at room temperature and for fourteen days when frozen at −20°C.

The applicability of the method was demonstrated by analysis of plasma samples from a male dog who received a single 10-mg oral dose of marplan in capsule form. The analytical method was validated in dog plasma prior to the analysis of samples from dosed dogs. There were no interfering peaks in any

TABLE I

INTER-ASSAY PRECISION FROM A CONSIDERATION OF THE CALIBRATION DATA

Fit of back-calculated concentrations to calibration line. $n=6$.

Marplan added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	R.S.D. (%)
100	97.7 ± 3.4	3.4
250	255.2 ± 7.5	2.9
500	498 ± 5.4	1.1
1000	1034 ± 55.6	5.4
2500	2463 ± 50.0	2.0
5000	5011 ± 50.6	1.0
Overall R.S.D.		2.6

TABLE II

INTRA-ASSAY PRECISION FROM A CONSIDERATION OF THE CALIBRATION DATA

Ratio of duplicate analyses.

Marplan added (ng/ml)	Ratio of determinations (mean ± S.D.)
100	1.016 ± 0.147
250	1.040 ± 0.055
500	1.088 ± 0.069
1000	1.028 ± 0.068
2500	1.010 ± 0.032
5000	1.013 ± 0.038
Low quality assurance	1.011 ± 0.020
High quality assurance	0.993 ± 0.019
Overall ratio mean ± S.D.	1.025 ± 0.056
Overall intra-assay precision	5.45%

TABLE III

BENCHTOP AND SHORT-TERM STABILITY OF MARPLAN ($n=5$)

Time	Temperature (°C)	Concentration of marplan (mean ± S.D.) (ng/ml)	R.S.D. (%)	Recovery (%)
0 h	Ambient	3610 ± 40.4	1.12	100
3 h	Ambient	3576 ± 39.0	1 09	99.1
6 h	Ambient	3526 ± 78.2	2.22	97.7
2 days	−20	3396 ± 67.0	1.97	94 1
14 days	−20	3500 ± 139.4	3.99	97.0

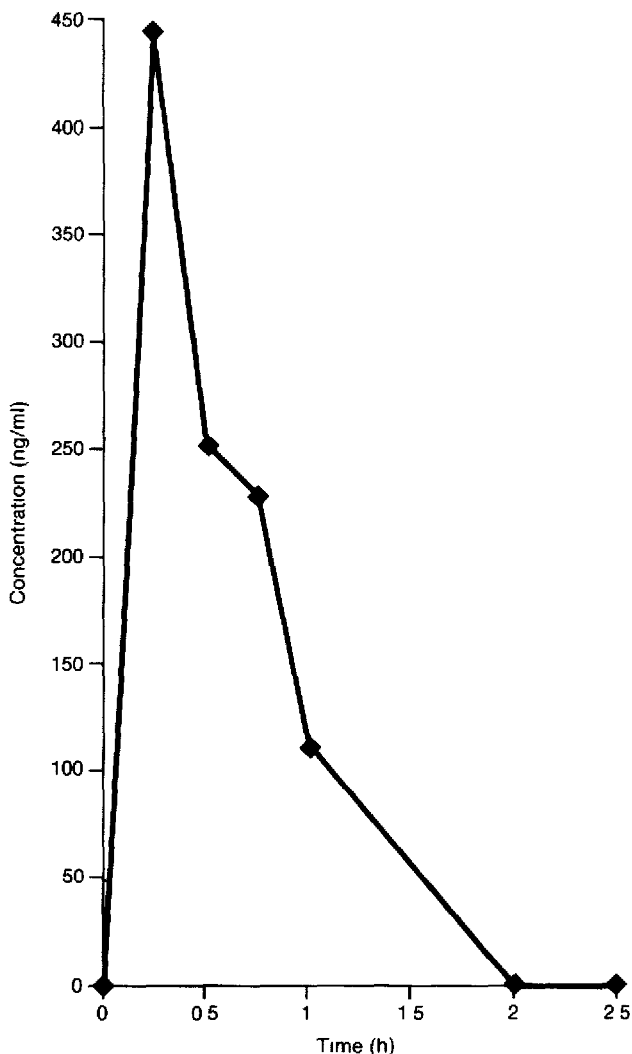

Fig. 3. Plasma level versus time curve for a male dog after a 10-mg oral dose of marplan.

dog plasma samples, and the inter- and intra-assay precision was 2.4 and 2.4%, respectively. The plasma concentration versus time curve for marplan is shown in Fig. 3. The drug was rapidly absorbed, achieving maximum plasma concentrations in less than 30 min, followed by rapid elimination.

CONCLUSIONS

A sensitive and specific HPLC assay has been developed for the analysis of marplan in human plasma. The limit of quantitation is 100 ng/ml using 0.5 ml

of plasma, and the assay was validated over a concentration range of 100–5000 ng/ml. The method may also be applied to samples from other species besides man.

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