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Note

Determination of paroxetine in human plasma, using high-performance liquid chromatography with fluorescence detection

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Paroxetine, (−)-*trans*-4-(*p*-fluorophenyl)-3-[[3,4-(methylenedioxy)-phenoxy]methyl]piperidine (I, Fig. 1), is a potent 5-hydroxytryptamine uptake inhibitor which is currently undergoing clinical evaluation for antidepressant efficacy in humans [1]. A gas chromatographic (GC) assay, using nitrogen-specific detection, has been reported [2] for the determination of paroxetine in human plasma, with a detection limit of 3 ng/ml. In order to follow plasma concentration–time profiles for up to three half-lives after a 30-mg oral dose, an assay was required with a minimum detection limit of ca. 0.5 ng/ml.

This paper describes a convenient, sensitive and reliable high-performance liquid chromatographic (HPLC) method for the determination of paroxetine, using maprotiline (II, Fig. 1) as internal standard and involving derivatization with dansyl chloride and subsequent fluorescence detection.

EXPERIMENTAL

Reagents

Methanol for the HPLC mobile phase, and toluene for the extractions, were both of HPLC grade (Baker); toluene was further glass-distilled prior to use. Dansyl chloride and L-proline were obtained from Sigma (Munich, F.R.G.). All other reagents were of analytical-reagent grade.

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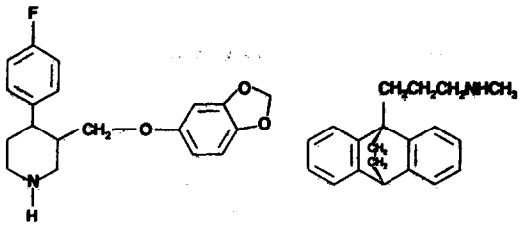

I. PAROXETINE **II. MAPROTILINE**

Fig. 1. Structural formulae of compounds I and II.

The internal standard used for this method, maprotiline (II, Fig. 1), was obtained from Ciba-Geigy (Horsham, U.K.).

Preparation of solutions

A 0.12 *M* phosphate buffer for the extraction of paroxetine from plasma was prepared by dissolving 8.6 g of disodium hydrogen orthophosphate dodecahydrate in 100 ml of water, bringing to pH 12.0 with 4 *M* sodium hydroxide and making up the final volume to 200 ml with distilled water.

For the aqueous component of the HPLC mobile phase, a pH 4.5 0.05 *M* sodium acetate buffer was prepared by dissolving 1.5 ml of glacial acetic acid in ca. 450 ml of water and bringing to pH 4.5 with 1 *M* sodium hydroxide. The solution was made up to a final volume of 500 ml with distilled water.

Stock aqueous solutions for both paroxetine and maprotiline were prepared at concentrations of 100 μg/ml (calculated as the pure free base). The paroxetine stock solution was diluted each day with distilled water to give a working solution of 100 ng/ml for preparation of the calibration samples. The final maprotiline concentration was dependent on the calibration range: a working solution of 25 ng/ml was suitable for a paroxetine range of 0–20 ng/ml.

The stock solutions of paroxetine and maprotiline remain usable for one week and three months, respectively, if stored at 4°C.

For the derivatization procedure, a 1 mg/ml solution of dansyl chloride in acetone, a 25 mg/ml solution of L-proline in water (both solutions prepared daily) and an aqueous solution of sodium hydrogen carbonate (0.1 *M*) were required.

Sample collection and storage

Blood for paroxetine assay was collected into EDTA tubes. These tubes were centrifuged (1500 g for 10 min) as soon as possible after collection and the resultant plasma separated and stored at ca. −20°C until assayed.

Sample preparation

Extractions were carried out in silanized glass tubes. These were prepared by overnight immersion in a 10% solution of Surfasil™ (Pierce, Rotterdam, The Netherlands) in toluene: the tubes were then thoroughly washed in toluene followed by methanol and oven-dried at 100°C.

To a series of 1-ml aliquots of control human plasma in silanized glass tubes

were added 250 μl of pH 12.0 phosphate buffer, 100 μl of internal standard solution and an appropriate volume (not exceeding 200 μl) of a freshly diluted paroxetine solution to yield concentrations in the range 0.25–20 ng/ml. Finally, 4 ml of glass-distilled toluene were added for the extraction.

Authentic samples were treated similarly. To 1 ml of plasma (in a silanized glass tube) were added 250 μl of pH 12.0 phosphate buffer, 200 μl of distilled water, 100 μl of internal standard solution and 4 ml of glass-distilled toluene.

The samples were extracted for 15 min on a tumble mixer and then centrifuged for 5 min at 1500 *g*. The upper toluene layer was transferred and evaporated to dryness under oxygen-free nitrogen using a sample concentrator at 55 °C.

Derivatization

To the resultant extract were added 50 μl of acetone, 25 μl of 0.1 *M* sodium hydrogen carbonate solution and 10 μl of dansyl chloride solution: each tube was capped, vigorously agitated by vortex mixer for 15 s and heated for 1 min at 55 °C in the sample concentrator. The tubes were briefly centrifuged (1 min) and left to stand at room temperature for 30 min. To remove excess derivatizing agent, 25 μl of L-proline solution were added, the tubes briefly agitated by vortex mixer and again centrifuged for 1 min. After standing for a further 5 min at room temperature, 500 μl of distilled water and 2 ml of glass-distilled toluene were added. Extraction of the now-derivatized compounds was carried out by agitation on a tumble mixer for 10 min, centrifugation for 5 min at 1500 *g*, transfer of the upper toluene layer and subsequent evaporation to dryness under oxygen-free nitrogen at 55 °C. The residue was redissolved in 100 μl of HPLC mobile phase, assisted by vortex mixing for 30 s. Where maximum sensitivity was required, the entire 100-μl sample was injected onto the column.

Chromatography

Chromatographic separation was achieved isocratically on a 20 cm × 4 mm I.D. Spherisorb 5-μm ODS column (Kontron, Zürich, Switzerland), fitted with a 3-cm guard column packed with the same material. Guard column and analytical column were connected using a cartridge system (Kontron). The HPLC system consisted of a Perkin-Elmer Series 2 pump, a Rheodyne 7105 loop injector, a Shimadzu RF 530 fluorescence spectromonitor and a Servogor chart recorder.

The mobile phase was prepared by mixing methanol (84% by volume) and pH 4.5 0.05 *M* sodium acetate buffer (16%). After filtering, this mixture was degassed under vacuum and delivered at a flow-rate of 1.5 ml/min. Excitation and emission wavelengths for the fluorimetric detector were optimized at 340 and 520 nm, respectively.

In extraction efficiency experiments for determining underivatized paroxetine, the HPLC system was modified by using a mobile phase of methanol–buffer (65:35), a μBondapak C_{18} column (Waters, Königstein, F.R.G.) and fluorescence detection with excitation and emission wavelengths of 300 and 350 nm, respectively.

Fig. 2. Chromatograms of (A) drug-free human plasma, (B) plasma from a subject receiving paroxetine (paroxetine concentration estimated at 4.1 ng/ml) and (C) human plasma spiked with paroxetine (10 ng/ml). Annotated peaks correspond to the dansyl derivatives of paroxetine (I, retention time 5.8 min) and the internal standard maprotiline (II, retention time 8.2 min).

Data analysis

Standard curves for paroxetine were constructed by plotting peak-height ratios (of paroxetine versus internal standard) against paroxetine concentration and fitting a straight line using unweighted least-squares regression. The fitted line was statistically evaluated using standard techniques [3], which have been adapted for use in our laboratories. The overall standard deviation of the regression is expressed in concentration terms (as the parameter λ) from the triangle of errors at the mean concentration. The precision of the assay is λ expressed as a percentage of the mean concentration. The parameter λ is then used to define a limit of reliable determination (LRD), the lower limit for accurate quantitation. The LRD is calculated as either 2λ (for a positive ordinate intercept) or 2λ plus the abscissa intercept (for a negative ordinate intercept). It thus approximates to a 95% confidence limit for concentration determination – estimated concentrations below the LRD cannot be distinguished from zero with greater than 95% confidence. The LRD is differentiated from the limit of detection, a parameter related to system performance and here defined as that amount of compound which gives a detector response twice that of the background noise level.

RESULTS

Typical chromatograms obtained using the method described above are shown in Fig. 2. Chromatograms illustrated were derived from control plasma (A), plasma from a subject receiving paroxetine hydrochloride (B) and plasma spiked

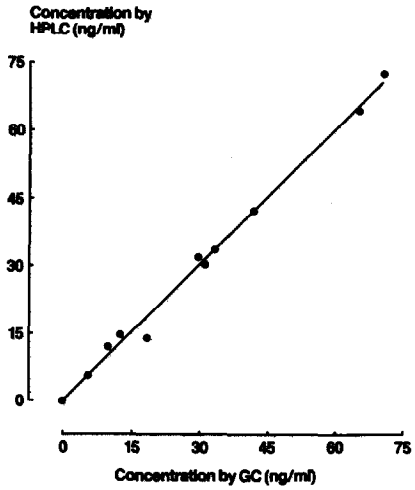

Fig. 3. Correlation between plasma concentrations of paroxetine determined by GC and HPLC in samples from patients receiving paroxetine. Slope = 1.02 ± 0.03; intercept = −0.57 ng/ml; correlation coefficient = 0.997.

at a level of 10 ng/ml (C). Both paroxetine and internal standard peaks are well resolved from endogenous material, with retention times of ca. 5.8 and 8.2 min, respectively. In the concentration range 20–120 ng/ml, absolute recovery from plasma ranged from 83 to 92%.

Precision, accuracy and sensitivity

The assay precision from individual standard curves is routinely within 5%; the within- and between-batch variability is generally below 10%. The limit of detection of this method is ca. 0.2 ng/ml, but LRD values are normally between 0.5 and 1 ng/ml for a 0–20 ng/ml calibration range.

Correlation studies

Correlation studies on duplicate plasma samples were carried out using both the HPLC method and the original GC method [2]. As seen in Fig. 3, acceptable agreement in the 5–100 ng/ml concentration range confirmed the equivalence of the two methods. Correlation between concentrations at less than 5 ng/ml was not possible, owing to the sensitivity limitation of the GC method.

Selectivity of the assay

There is usually no interference with the assay arising from endogenous substances; a very small peak has on occasions been observed from control plasma, eluting at the same time as paroxetine but equivalent to less than 0.2 ng/ml (expressed in terms of paroxetine concentration). Known metabolites do not interfere with the quantitation of paroxetine itself.

Interaction studies in humans have been performed with a number of other drugs, including phenytoin, phenobarbitone, methyldopa, cimetidine, digoxin,

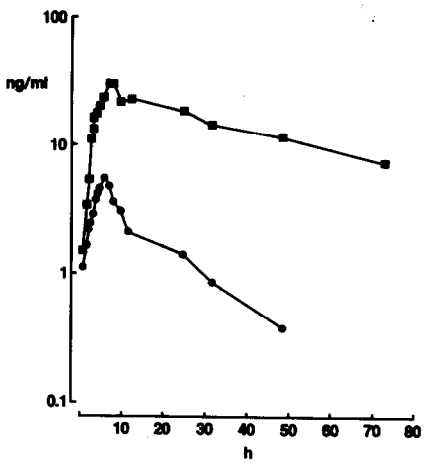

Fig. 4. Plasma concentrations of paroxetine in normal healthy volunteers after the oral administration of 30 mg of paroxetine (as the hydrochloride salt).

procyclidine and tranylcypromine. None of these drugs has been found to interfere with the assay of paroxetine.

Assay of paroxetine in plasma samples

The assay for the determination of paroxetine in human plasma has been used routinely for ca. eighteen months. The data presented in Fig. 4 exemplify the range of human profiles for which the method has utility. In these two volunteers maximum observed plasma concentrations were 5.5 and 30.0 ng/ml, declining to ca. 0.5 and 9.0 ng/ml at 48 and 72 h, respectively.

DISCUSSION

The data presented here have emphasized the application of the method to the assay of paroxetine in plasma in the 0–20 ng/ml concentration range. However, the method may be equally applied where concentrations of paroxetine are higher, and linear calibration curves have been constructed up to 500 ng/ml. Following oral doses of 30 mg of paroxetine (as the hydrochloride salt), the majority of subjects show plasma levels up to 20 ng/ml. After higher or repeat dose regimens, however, plasma levels over 100 ng/ml have been observed.

A large number of samples from a wide variety of clinical studies have been successfully assayed, thereby demonstrating the robustness of the method in routine application. During this time, experience with the method has revealed a number of points which should be noted. The separation of the peaks of interest from the endogenous background is critically dependent on the proportion of methanol to buffer in the mobile phase; the optimum mobile phase composition should be established for each column prior to assay work commencing.

Following the completion of a batch of sample analyses, it is advisable to wash the column with a mobile phase in which the methanol content has been increased

to 95%. This rapidly removes retained material and prepares the column for undisturbed use on the following day.

Finally, the use of silanized tubes is only beneficial for the initial extraction stage. Subsequently, methanol-rinsed oven-dried glassware should be used. It has been shown that derivatization with dansyl chloride will not proceed satisfactorily in silanized tubes.

The method has been successfully transferred to other laboratories. In particular, using a Model 710B WISP autoinjector (Waters) with a Model 510 pump (Waters), a Model 3000 fluorescence spectrometer (Perkin-Elmer) and a Multichrom data acquisition system (VG Laboratory Systems), chromatographic separation and quantitation have been successfully automated.

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