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Short Communication

High-performance liquid chromatographic method for the determination of a potential memory-enhancing compound (CL 275,838) and its desbenzyl metabolite in rat plasma or serum

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ABSTRACT

A rapid, selective, precise reversed-phase liquid chromatographic method has been developed for the determination of a potential memory-enhancing agent (CL 275,838) and its main metabolite (CL 286,527) in plasma and serum. The procedure includes isolation of compounds from proteins precipitated with acetonitrile, subsequent resolution by reversed-phase (Whatman Partisphere C_8) high-performance liquid chromatography and ultraviolet detection. The assay was linear over the range 0.12–1.25 μg/ml of plasma or serum. The detection limit was 0.12 μg/ml, using 0.2 ml of plasma or serum. Intra- and inter-day validation studies indicated an acceptable precision and reproducibility of the method within the concentration range investigated, the overall coefficient of variation being less than 10%. The method is currently applied in support of pharmacological and toxicity studies of the compound in rodents.

INTRODUCTION

Compound CL 275,838 (hereafter referred to as I), 4,5-dihydro-4-[[4-(phenylmethyl)-1-piperazinyl]acetyl]-7-[3-(trifluoromethyl)phenyl]pyrazolo[1,5-*a*]pyrimidine-3-carbonitrile dihydrochloride, is a potential memory-enhancing drug currently undergoing preclinical pharmacological and toxicological studies. Over the past twenty years there has been continued interest in developing drugs capable of enhancing memory and learning ability in animals, and a number of compounds, broadly classified as cognition enhancers, have been or are shortly to be introduced in the hope of providing a treatment for senile dementia [1–3]. Chemically, compound I is unrelated to all these known agents with similar indications (see also Fig. 1). Pharmacologically, it shows activity in a wide series of *in vivo* tests predictive of age-related neurobehavioural dysfunctions while presenting a unique biochemical profile and no measurable toxicity even at doses higher than the effective range [4].

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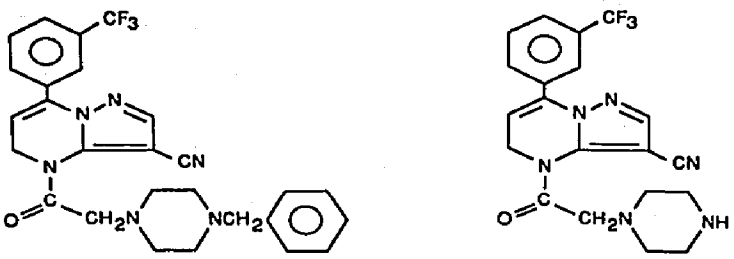

(I) (II)

Fig. 1. Structures of compound I (CL 275,838) and its desbenzyl metabolite (II).

Early compound I disposition studies conducted with radiolabelled techniques have provided most of our knowledge of the kinetics and metabolism of this compound in animals. Compound I appears to be efficiently extracted by the liver, and its systemic clearance is essentially due to metabolism. The main metabolite is the desbenzyl derivative, 4,5-dihydro-4-[(1-piperazinyl)acetyl]-7-[3-(trifluoromethyl)phenyl]pyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (II, Fig. 1), which appears in significant amounts in animal tissues and urine [5].

As during the current stage of drug development the need arose for a rapid procedure to provide information on the circulating concentrations of the parent drug and its main metabolites associated with the doses employed in pharmacological and toxicity studies, we developed the present chromatographic method to detect I and its desbenzyl metabolite in rat plasma and serum.

EXPERIMENTAL

Reagents and solvents

The parent compounds and its main metabolite were provided by Cyanamid-Italia (Catania, Italy). The internal standard (I.S.), 4,4-methylethyl-1-[4-(pyrimidinyl)-1-piperazinyl)butyl]-2,6-piperidinedione hydrochloride, was kindly supplied by Mead Johnson (Evansville, IN, USA).

Acetonitrile for extraction and high-performance liquid chromatography (HPLC) was HPLC-grade and was obtained from Omnia Res (Milan, Italy). Other chemicals (potassium dihydrogenphosphate, phosphoric acid) (Merck, Darmstadt, Germany) were of analytical-reagent grade and were used without further purification.

Standard solutions

Stock solutions of I, its metabolite II and the I.S. were prepared weekly in acetronitrile at a concentration of 1 mg/ml. Standard solutions (1 and 10 μg/ml) were prepared from stock solutions by dilution with acetonitrile.

Apparatus and chromatographic conditions

A Waters system (Waters Assoc., Milford, MA, USA) was used equipped with

a Model U6K universal liquid injector, a Model 6000A solvent delivery system and a Model 440 UV detector monitor at 254 nm, a reversed-phase octadecyl silica column (Whatman Partisphere C_8, 12.5 cm × 4.6 mm I.D.) (Farmitalia Carlo Erba, Milan, Italy), maintained at room temperature, and a guard column filled with the same packing. The mobile phase was acetonitrile–0.01 *M* potassium dihydrogenphosphate (52:48, v/v) adjusted to pH 7.0 with concentrated phosphoric acid. The flow-rate was set at 1.2 ml/min.

Sample preparation

The sample preparation procedure for the analysis of I and II in rat plasma or serum involved protein precipitation using 0.2 ml of sample and 1.6 ml of acetonitrile containing the I.S. (0.1 μg). After centrifugation, the clean supernatant was evaporated to dryness *in vacuo*. The residue was dissolved in 0.1 ml of the mobile phase and 90 μl were injected into the HPLC system. Concentrations of the parent compound and its metabolite were calculated by the internal standard method using multi-point ($n = 5$) linear calibration.

Stability studies

To verify the stability of the parent compound and its desbenzyl metabolite in plasma and serum before extraction, portions of the standard solutions of the two compounds were spiked (25 and 250 ng) into extraction tubes containing fresh rat plasma (heparinized) or serum (0.2 ml). The tubes were stored at room temperature or –20°C before analysis. The tubes at room temperature were analysed at various intervals up to 24 h, and those at −20°C were analysed one month later to check the long-term stability of the two compounds.

Validation procedure

The precision and reproducibility of the method were determined by replicate analysis ($n = 6$) of rat plasma (0.2 ml) containing I and II at concentrations of 0.125–1.25 ng/ml. The inter-assay precision and accuracy were evaluated by preparing quality control samples (QC) at the start of the validation study: with each day's analysis, these QC standards were assayed with standard samples and the calculated concentrations were compared. Intra-assay precision was checked by replicate analysis of QC samples on the same days.

Studies in rats

These studies were conducted in conjunction with long-term toxicity studies of compound I in rodents (Cyanamid-Italia). Male and female Sprague–Dawley rats (Charles River, Calco, Italy) weighing 100–125 g, age 4–5 weeks, were given 50–300 mg/kg I orally in 2% succinyl gelatine ± 0.1% keltrol (xanthan gum) in 0.1 *M* tribasic sodium citrate and were killed at various times (0–6 h) thereafter to collect adequate volumes of serum. Time to peak (t_{max}) was defined as the sampling time when maximal mean concentrations (C_{max}) were found. The area under

the curve (AUC) from zero to the last measurement time was calculated according to a standard procedure [6].

RESULTS AND DISCUSSION

Initial efforts to isolate compound I and its metabolite II from biological fluids with organic solvents such as ethyl acetate, chloroform, hexane and benzene were not satisfactory in terms of recovery and/or specificity. It was then found that both compounds could be rapidly and reproducibly isolated from rat plasma or serum constituents with acetonitrile. With this extraction procedure, recovery, determined by comparing the peak heights of I and II after injection of non-extracted standard solutions and after injection of extracted plasma or serum containing 0.125, 0.5 and 1.25 ng/ml of both compounds, was, respectively, 80 ± 5, 79 ± 4 and 77 ± 7% and 78 ± 3, 76 ± 2 and 70 ± 3%; coefficients of variation (C.V.) were between 3 and 10% for the parent compound and its main metabolite.

Fig. 2 illustrates typical chromatograms of extracts from (a) drug-free plasma and (b) plasma of a rat treated orally with compound I. The chromatographic peaks have baseline separation, and the extract of drug-free plasma shows no peaks that could interfere with analysis of the parent drug or its metabolite. Compound I eluted at 9.7 min, the metabolite at 6.9 min and the I.S. at 4.7 min, as shown by injection of non-extracted standard solutions of the compounds.

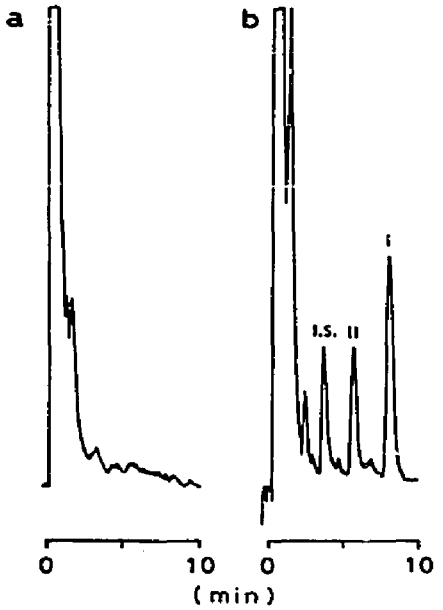

Fig. 2. Chromatograms of extracts from (a) blank serum and (b) serum sample (0.2 ml) of a female rat treated orally with compound I (300 mg/kg) containing (30 min after dosing) 0.44 μg/ml parent compound and 0.43 μg/ml desbenzyl metabolite (II). I.S. = internal standard.

Following the procedure described above, the stability of I and II was first evaluated under different conditions of temperature and storage times. No significant decrease of concentrations was detected when the parent compound and its metabolite were stored in plasma or serum for 1–2 h at 23°C. However, degradation of I in both fluids was observed 4 h later, becoming particularly evident (a more than 80% drop from the initial concentration) after 24 h of storage. These data clearly indicated that the maximum time for handling rat plasma and serum samples at room temperature should be limited to 2 h. Neither I nor its metabolite II revealed any instability during storage in the refrigerator for one month.

The graphic relationships between the peak-height ratio of I and II to I.S. and the amount of the compounds added to plasma or serum were always linear for the plasma concentration range 0.125–1.25 μg/ml. The correlation coefficient for each standard curve plotted invariably exceeded 0.99; the slopes of six curves, prepared throughout the day-to-day validation study, had a C.V. of 9 and 7% for the parent drug (average regression equation $y = 0.005x - 0.008$) and the metabolite ($y = 0.004x + 0.010$), respectively. The limit of detection was approximately 0.125 μg/ml for both compounds, using 0.2 ml of plasma. This limit of detection was more than adequate for our purpose because the concentrations in rat plasma or serum within the range of doses employed in preliminary pharmacological and toxicological studies were generally higher than the sensitivity limits within 6 h of parent drug administration. Rat plasma QC samples (0.2 ml) containing the equivalent of 25, 100 and 250 ng of I and II were assayed ($n = 4$) with each of the HPLC chromatographic runs in support of this study. For the analysis of I in rat plasma mean C.V.s were, respectively, 8.3, 4.2 and 3.3% at 0.125, 0.5 and 1.25 μg/ml for six experiments on six different days. Overall mean C.V.s for the metabolite II were, respectively, 9.1, 6.1 and 3.3% for the 0.125, 0.5 and 1.25 μg/ml QC samples for six different experiments and four samples at each metabolite level and experiment.

Actual concentrations found in these samples were, with added concentrations in parentheses, 0.12 (0.125), 0.48 (0.5) and 1.23 (1.25) μg/ml for compound I and 0.11 (0.125), 0.49 (0.5) and 1.22 (1.25) μg/ml for its desbenzyl metabolite II. The overall mean accuracy of the method calculated from the deviation of the mean concentration found from the nominal value of each compound indicated an inter-assay variation in the range from −1.6 to −4% for I and −12 to −2% for its metabolite II. Thus the precision and accuracy of the method appear to be acceptable over the concentration range investigated.

The analytical procedure is currently used in our laboratory to measure the plasma or serum concentrations of the parent compound and its desbenzyl metabolite associated with the doses utilized in long-term toxicity studies in rodents. No differences were observed between serum and plasma samples spiked with known amounts of I and II. Mean serum concentration–time profiles of the parent drug and its desbenzyl derivative on day 0 of these studies are presented in Fig. 3. It appears that oral doses of I (50, 150 and 300 mg/kg) were rapidly

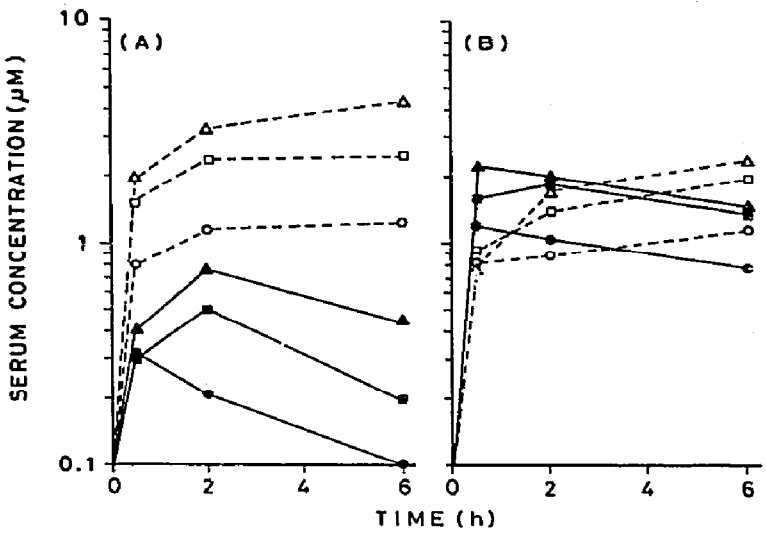

Fig. 3. Serum concentration–time curves of compound I (closed symbols) and its desbenzyl metabolite (open symbols) in male (A) and female (B) rats given oral CL 275,838. Doses of I (in terms of free base) were: 50 (●,○), 150 (■,□) and 300 (▲,△) mg/kg.

absorbed from the rat gastrointestinal tract, achieving mean peak serum concentrations within 0.5–2 h of administration regardless of the dose and sex. However, females had higher C_{max} and AUC values than male rats. The metabolite mean C_{max} and AUC were similar to (female) or higher than (male) those of the parent drug, but in general did not show noteworty sex differences. Exposure to the drug and its main metabolite was to some extent dose-related when referred to mean serum C_{max} and AUC, although the proportionality was closer in male than in female rats.

In conclusion, the method proposed is selective for I and II and has a limit of detection of 0.125 μg/m with an overall mean C.V. and accuracy error of less than 10% for both the parent compound and its metabolite. The serum volumes required are small and the extraction is rapid and simple. In terms of analysis time, specificity and sensitivity the procedure therefore appears adequate to measure the drug and metabolite plasma and serum concentrations which might be met in pharmacological and toxicity studies.

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