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Liquid chromatography of the potential memory-enhancing agent CL 275,838 and its main metabolites, using a post-column photochemical reactor and fluorimetric detection

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ABSTRACT

On irradiation with short-wavelength ultraviolet light, the potential memory-enhancing compound CL 275,838 (I) and its desbenzyl derivative CL 286,527 (metabolite II) are cleaved into the highly fluorescent derivative CL 228,346 (metabolite IV). This reaction was exploited for the sensitive and selective detection of these compounds in human and animal plasma, after reversed-phase high-performance liquid chromatography on a Supelco LC18 DB column (15 cm × 4.6 mm I.D.) at room temperature. The parent compound and its metabolites were isolated from plasma constituents using the Sep-Pak C_{18} Plus cartridge, with satisfactory recovery (76–90%) and selectivity. The detection limits were *ca.* 1.25, 5 and 0.3 ng/ml for I, II and IV, respectively, using 1 ml of plasma. The validation procedure, which includes analysis of multiple ascending calibration curves based on between-day values and replicate analysis of quality control samples analysed with each standard curve, indicated acceptable precision and accuracy of the method within the concentration ranges investigated, the overall coefficient of variation and relative error being less than 10%. The method was successfully applied to plasma samples from healthy volunteers and animals after single of multiple doses of compound I. Metabolites II and IV were detectable in plasma of all species, the former at higher concentrations than the parent compound and metabolite IV. Together with the fact that metabolite II retains much of the parent compound's biological activity *in vivo* and *in vitro*, this suggests that it may contribute to the pharmacological effects of compound I.

INTRODUCTION

Over the past twenty-odd years considerable research effort has been expended on identifying new agents capable of enhancing memory and learning ability in animals, in the hope of developing more effective drugs for such therapies (see refs. 1–3 for reviews). One such agent is compound CL 275,838 (I) or 4,5 dihydro-4-[[4-(phenylmethyl)-1-piperazinyl)acetyl]-7-[3-(trifluoromethyl)phenyl]pyrazolo[1,5-*a*]pyrimidine-3-carbonitrile dihydrochloride, which is structurally (Fig. 1) and pharmacologically unrelated to any existing drugs with similar pharmacological properties. The compound shows activity in animal models indicative of antidepressant activity [4,5]. It is currently undergoing extensive clinical

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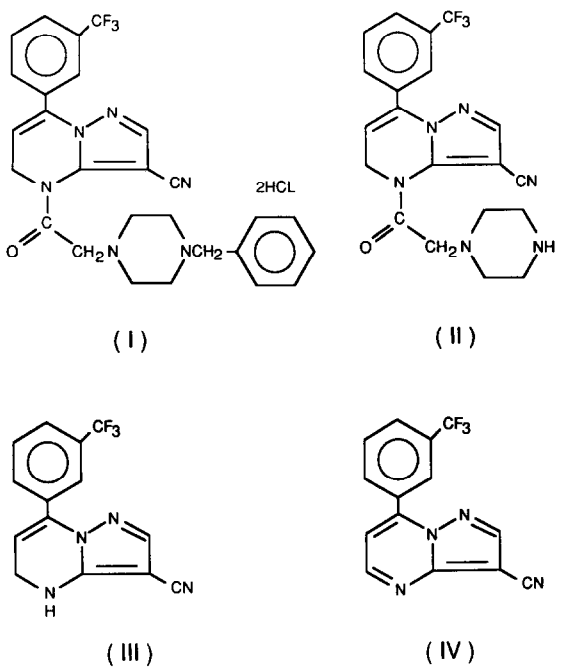

Fig. 1. Structures of compound I and its metabolites II, III and IV.

trials to test safety and clinical efficacy. Measurement of its blood concentrations is certainly helpful in such studies.

Compound I is rapidly and extensively biotransformed in all the species so far investigated. Its biotransformation occurs by two initial major pathways, the desbenzyl derivative CL 286,527 (metabolite II) and hippuric acid being the major metabolites excreted in urine. Other urinary metabolites include CL 273,352 (metabolite III), its oxidized derivative CL 228,346 (metabolite IV) and CL 181,783 (metabolite V). In peripheral circulation and tissues, however, most of the radiolabel was accounted for by metabolite II, with quantitative differences between species [6]. This metabolism occurs in human too [7]. Concentrations of metabolite II should thus be included for measurement in any clinical studies of compound I, and in this respect a selective and sensitive chromatographic procedure for the simultaneous determination of the parent compound and its main metabolite would be advantageous.

Compound I has been quantitated in body fluids not only by radiolabeling techniques but also by high-performance liquid chromatography (HPLC) with either UV detection at 254 nm [8] or post-column oxidation of the eluate to form a derivative that can be measured by its fluorescence [9,10]. On account of the extensive biotransformation and tissue distribution of I, the conventional HPLC–UV method, which included measurement of metabolite II, is not sensitive enough to quantitate plasma concentrations of the parent compound after therapeutic doses [8]. Adequate sensitivity was achieved using post-column derivatization or photolysis with fluorescence detection, but these methods did not include the measurement of the desbenzyl derivative II [9,10].

As part of the effort to develop the drug, we have therefore set up a relatively simple yet highly selective HPLC procedure with fluorescence detection, which is sufficiently sensitive to measure the plasma concentrations of compound I and its metabolites II and IV that might be met in clinical studies. The procedure relies on solid-phase extraction of the compounds, resolution on a reversed-phase column and post-column photolysis to convert the parent compound and its main metabolite II into the highly fluorescent derivative IV. The use of selective photochemical reactors for post-column derivatization has been recently reviewed [11].

EXPERIMENTAL

Chemicals

Compound I, its metabolites 4,5-dihydro-4-[[1-(piperazinyl]acetyl]-7-[3-(trifluoromethyl)-phenyl]pyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (II), 4,5-dihydro-7-[3-(trifluoromethyl)phenyl]-pyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (III), 7-[3-(trifluoromethyl)phenyl]pyrazolo[1,5-*a*]-pyrimidine-3-carbonitrile (IV) and pyrazolo[1,5-*a*]-pyrimidine-3-carbonitrile-4,5-dihydro-5-oxo-7-[3-(trifluoromethyl)phenyl] (V) and the internal standard (I.S.), 5-methyl-7-[3-(trifluoromethyl)-phenyl]pyrazolo[1,5-*a*]pyrimidine-3-carbonitrile, were of pharmaceutical grade, supplied by American Cyanamid Company (Pearl River, NY, USA).

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

Acetonitrile for extraction was HPLC grade and was obtained from Omnia Res (Milan, Italy). Other chemicals (methanol, monosodium phosphate, Farmitalia–Carlo Erba, Milan, Italy; phosphoric acid, E. Merck, Darmstadt, Germany) were of analytical-reagent grade and were used without further purification.

Sample preparation

The sample preparation procedure was a modification of the previously developed solid-phase extraction of compound I from human plasma [9]. Briefly, the Sep-Pak C_{18} Plus (360 mg) cartridge (Waters Assoc., Milford, MA, USA) was pre-wetted with 5 ml of acetonitrile, 5 ml of acetonitrile–water (50:50, v/v) and 5 ml of distilled water. Then 1–2 ml of human plasma, or 0.2–1 ml of rat or dog plasma (containing 2.5 ng of I.S.), were added, and the cartridge was washed with 5 ml of distilled water, 5 ml of acetonitrile–water (20:80, v/v), 1 ml of 0.01 *M* hydrochloric acid (which allows the subsequent extraction of the desbenzyl derivative II) and 0.35 ml of acetonitrile. The compounds of interest were removed by eluting the cartridge with 2 ml of acetonitrile, then evaporated to dryness *in vacuo*. The residue was dissolved in 0.2 ml of the mobile phase and centrifuged in Eppendorf polypropylene tubes at 12 000 *g* for 5 min, and the clean supernatant was set in a sample tray for automatic injection and analysed by HPLC using post-column derivatization with fluorescence detection. A 190-μl sample was used for each analysis.

Instrumentation

A Waters system equipped with a Wisp-712 sample processor and a Model 510 solvent-delivery system (Waters Assoc.) were used. A reversed-phase column (Supelco LC 18 DB, 15 cm × 4.6 mm I.D., 5 μm particle size), maintained at room temperature, and a Newguard RP 18 precolumn (7 μm particle size, Brownlee Labs., Dupont, Santa Clara, CA, USA) were fitted. The mobile phase was acetonitrile–methanol–0.1 *M* NaH_2PO_4–0.02 *M* H_3PO_4 (22:26:26:26) delivered isocratically at a flow-rate of 1.0 ml/min. The mobile phase was filtered through a 0.45-μm filter and degassed under vacuum before use.

The column eluate was led into a photochemical reaction unit "Beam Boost" (International Chromatography Technology, Frankfurt, Germany). Photooxidation was accomplished in a reaction coil (10 m × 0.3 mm I.D.) made of an inert fluorinated polymeric material and coiled around the UV lamp (254 nm). The irradiation time was 60 s. A Shimadzu RF 530 fluorescence monitor was used at an excitation wavelength of 335 nm, and a Corning CS3-73 sharp-cut filter (cut wavelength 416–436 nm) was used in the emission path. The detector was coupled to a C-R6A Chromatopac Shimadzu integrator (A.STR.AN, Shimadzu) for the determination of peak heights.

Validation procedure

Drug-free plasma containing known amounts of compound I (1.25–25 ng/ml), metabolites II (2.5–100 ng/ml) and IV (0.31–12.5 ng/ml) were analysed concurrently with each set of quality control (QC) samples. Standard calibration curves were constructed by linear least-squares regression analysis of the plot of the peak-height ratios between the two compounds and the I.S., against their concentrations in biological samples (internal standard method).

The intra-assay precision across the working range was checked by replicate analysis of QC samples on the same day. The inter-assay precision was checked by preparing QC samples containing small, medium and large amounts of the parent compound and its metabolites at the start of the validation study. With each day's analysis, these QC samples were assayed with standard samples, and the calculated concentrations were compared.

Daily standard curves with six concentrations across the working range were measured in duplicate, with QC samples injected between the two sets of standards. The concentrations of com-

pound I and its metabolites in the QC and biological samples were determined by interpolation from the calibration curves using peak-height ratios obtained from the samples.

Application

Studies in healthy human subjects. Plasma samples were obtained from male volunteers, aged between 20 and 43 years and within 15% of normal body weight, with normal clinical and biochemical profiles who received single (50- or 100-mg tablets) and multiple doses (50 or 100 mg daily for two weeks) of compound I orally. Each subject was given a full explanation of the purpose of the study and signed an informed consent form approved by the local ethical committee. The study was conducted at Clinica Zucchi (Monza, Italy). A venous blood sample was drawn before (time 0) and at various times after the first and last doses. During the multiple-dose schedule "trough" blood samples were collected before each morning dose on days 4, 7, 10 and 14. Each sample was processed to produce plasma, which was stored frozen (−20°C) until analysis. Model-independent methods [12] were applied for the pharmacokinetic analysis of the parent compound and its metabolites.

Studies in animals. Compound I was administered orally to male CD-COBS rats, weighing *ca.* 300 g (Charles River, Como, Italy), and male beagles (11–15 kg) (S. Morini, S. Polo D'Enzo, Italy) at the lowest doses used in long-term toxicity studies (50 and 2 mg/kg for rats and dogs, respectively). Blood specimens were obtained by venipuncture (dog) or through a cannula previously implanted in the jugular vein (rat) at preset times from 15 min to 24 h after administration of compound I.

RESULTS AND DISCUSSION

Fluorescence detection

Compound I (which shows no native fluoresence) is rapidly transformed by short-wavelength UV radiation into a derivative, IV, which can be measured by its strong fluorescence with maximum emission at 416–436 nm [9]. The same reaction occurs under alkaline conditions, probably involving hydrolysis of the acyl group of compound I at position 4 of the pyrazolopyrimidine moiety, followed by oxidation of the product of hydrolysis [10]. Similar photooxidation and rapid reaction were seen for metabolites II and III, whereas V was apparently not converted into any fluorescent species. This, and the fact that the extraction characteristics of metabolite V differed from the other compounds (see below), precluded its simultaneous determination in human plasma. Metabolite III, on the other hand, is unstable, being converted spontaneously into IV, so this putative metabolite could not be determined either.

Chromatograms of the photolytic decomposition product of the parent compound and its putative metabolites using post- (A) and pre-column (B) photolysis and fluorescence detection is shown in Fig. 2. The peak of the fluorescent metabolite IV was identified on the basis of the chromatographic retention time of the authentic compound.

Sample preparation and chromatography

A Sep-Pak C_{18} Plus cartridge was used to

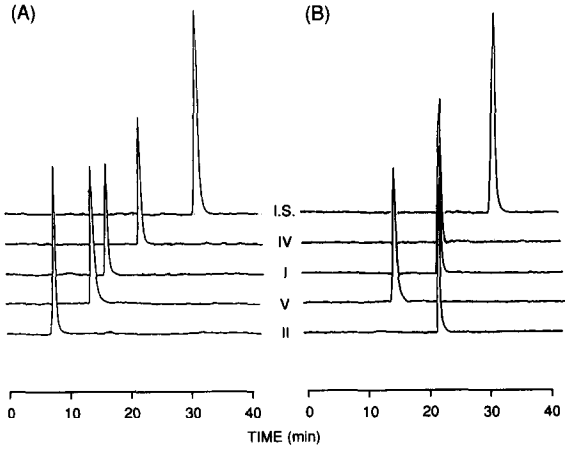

Fig. 2. Chromatograms of the photolytic decomposition product of compound I (10 ng amount injected) and its metabolites II (10 ng), IV (2 ng) and V (50 ng) using (A) post- and (B) pre-column photolysis and fluorescence detection. I.S. = internal standard.

clean up plasma samples. The parent compound and its metabolites II and IV could be isolated simultaneously on these cartridges by applying the sample at physiological pH and using a dilute hydrochloric acid solution and subsequent elution with acetonitrile. As mentioned above, however, this procedure precluded the simultaneous extraction of the putative metabolite V (retention time 14 min) because its recovery was so inconsistent.

Under these conditions the overall mean recovery, determined by comparing the peak-height ratios measured in extracts of human plasma spiked with the parent compound (1.25–25 ng/ml) and its metabolites II (5–100 ng/ml) and IV (0.31–6.25 ng/ml) with the peak-height ratios for acetonitrile-diluted compound standards, was 76 ± 7% for compound I and 81 ± 8 and 90 ± 7% for metabolites II and IV, respectively, with no significant dependence on concentration over the range investigated. Results were similar in extracts of rat plasma (0.2–1 ml) and dog plasma (0.5–1 ml), the recovery and selectivity both being relatively unaffected (data not shown).

Examples of chromatograms of extracts from a drug-free plasma and plasma from a healthy volunteer given 50 mg of compound I orally and containing 3 ng/ml compound I, 18 ng/ml metabolite II and 0.4 ng/ml metabolite IV, are shown in Fig. 3. Approximate retention times were 7.8 min for metabolite II, 16.5 min for compound I, 22 min for metabolite IV and 31.4 min for the I.S. We observed no interfering peaks in several samples of drug-free plasma from healthy subjects and patients. No interferences were found when drug-free plasma spiked with all the four compounds and some common anxiolytics (diazepam, nordiazepam, flurazepam, chlordiazepoxide, oxazepam, buspirone), antidepressants (imipramine, trazodone, fluoxetine), antipsychotics (haloperidol, chlorpromazine, prochlorperazine) and other drugs that might be administered to elderly patients (propranolol, atenolol, cimetidine, amiodarone, prazosin, flecainide) were processed at concentrations typically encountered in clinical samples according to this procedure.

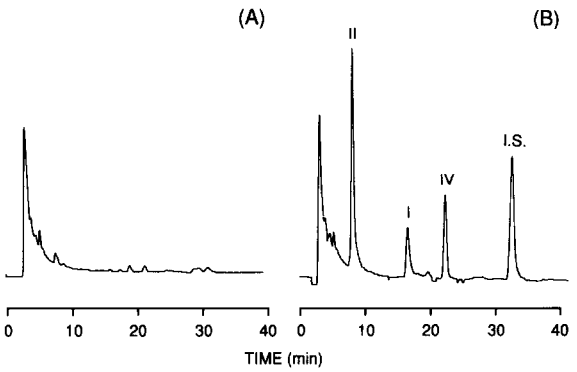

Fig. 3. Chromatograms of extracts from (A) drug-free plasma and (B) plasma from a volunteer given a 50-mg tablet of compound I and containing (24 h after dosing) 3 ng/ml unchanged compound, 18 ng/ml metabolite II and 0.4 ng/ml metabolite IV. I.S. = internal standard (2.5 ng/ml).

Chromatograms of drug-free extracts from rat plasma (A) and dog plasma (C) are shown in Fig. 4. As with human plasma, there are no peaks that could interfere with analysis of the parent compound and its main metabolites. Also shown are chromatograms of the plasma extracts (1 h after dosing) from a rat (B) and a dog (D) given compound I orally; in both species the parent compound (25 and 16 ng/ml in rat and dog, respectively) was rapidly and extensively biotransformed to metabolite II (45 and 46 ng/ml, respectively) and to a lesser degree to metabolite IV (9 and 9 ng/ml, respectively).

Validation studies

The relationships between the peak-height ratios of I, II and IV to the I.S. and the amount of the compound added to plasma were always linear, with a mean coefficient of correlation (r) approaching unity for the parent compound (r = 0.998 ± 0.003, n = 6) and its metabolites II (0.996 ± 0.005) and IV (0.998 ± 0.003). The slope of these curves, prepared over a period of three weeks, ranged from 0.088 to 0.091 (mean 0.0890 ± 0.0009) for the parent compound (average regression equation: $y = 0.089x + 0.002$), 0.150 to 0.154 (mean 0.1522 ± 0.0021) for metabolite II ($y = 0.152x + 0.094$) and 0.622 to

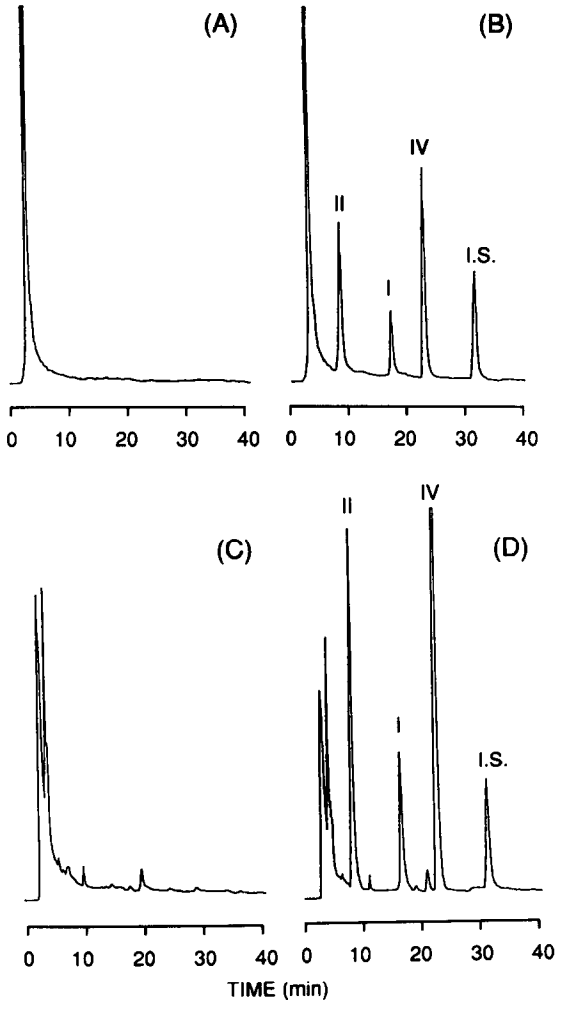

Fig. 4. Chromatograms of extracts of drug-free plasma samples (A, 0.2 ml; C, 0.5 ml) and plasma of rats and dogs treated with compound I (50 mg/kg and 2 mg/kg, respectively) containing (1 h after dosing) 25, 45 and 9 ng/ml and 16, 46 and 9 ng/ml of the parent compound and its metabolite II and IV, respectively, in the rat (B) and dog (D).

0.694 (mean 0.6578 ± 0.0305) for metabolite IV ($y = 0.658x + 0.031$). The lower limit of quantitation (the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision) was approximately 1.25, 5 and 0.3 ng/ml for I, II and IV, respectively, using 1 ml of plasma. This limit can be reduced still further (0.63, 2.5 and 0.15 ng/ml for the parent compound and its two metabolites, respectively) by using 2 ml of plasma. At these concentrations the coefficient of variation (C.V.) was 4.3% for the parent compound and 14.6 and 5.6% for metabolites II and IV, respectively. For all higher concentrations the C.V. was less than 10% for all compounds. Previous studies have established that compound I is sufficiently stable in human plasma for up to 24 h at room temperature and up to three months at −20°C [10]. However, degradation of I and II occurs in rat plasma only 4 h after storage at 23°C, indicating that the maximum time for handling rat samples at room temperature should be limited to 2 h [8].

Human plasma QC samples containing the equivalent of 1.25–25 ng/ml compound I, 2.5–100 ng/ml metabolite II and 0.31–12.5 ng/ml metabolite IV were assayed with each of the HPLC chromatographic runs in support of this study. The mean C.V. of intra-assay precision was 3.1–6.2% for the parent compound and 3.2–8.2 and 3.8–5.9% for metabolites II and IV, respectively (Table I). The mean C.V. of inter-assay precision (Table II) indicate that the method had good reproducibility, the C.V. being less than 10% at all concentrations tested. The relative error (R.E.) of these QC samples, $(F - A)/A \times 100$, calculated using the deviation of the concentration found (F) from the nominal value (A), indicated inter-assay variation from 0.8 to 6.4% from −1.6 to 1.6% and from 0.8 to 9.7%, respectively, for compound I and its metabolites II and IV.

Clinical applications

The utility of the analytical procedure was established by analysing plasma samples from healthy male volunteers who received single and multiple oral doses of compound I as tablets. Mean plasma concentration–time curves of compound I and its metabolites II and IV after a single oral dose of the drug (50 mg) are shown in Fig. 5. Peak plasma concentrations (C_{max}) of compound I were reached within 4 h of dosage in all subjects ($n = 10$). The mean C_{max} was 21 ng/ml but varied considerably between subjects (from 3 to 45 ng/ml). The C_{max} of metabolites II and IV ranged from 5 to 39 ng/ml (mean 18 ng/

TABLE I

SUMMARY OF QUALITY CONTROL RESULTS FOR COMPOUND I AND ITS METABOLITES II AND IV IN HUMAN PLASMA FROM THE WITHIN-DAY VALIDATION STUDY

Results are mean and S.D. (n = 4).

Concentration added (ng/ml)	Concentration found (ng/ml)	Coefficient of variation (%)	Accuracy (%)
Parent compound			
1.25	1.29 (0.08)	6.2	3.2
5	4.87 (0.17)	3.5	−2.6
25	24.85 (0.77)	3.1	−0.6
Metabolite II			
5	4.99 (0.41)	8.2	−0.2
20	20.02 (0.81)	4.1	0.1
100	99.11 (3.21)	3.2	−0.9
Metabolite IV			
0.31	0.34 (0.02)	5.9	9.7
1.25	1.28 (0.05)	3.9	2.4
6.25	6.09 (0.23)	3.8	−2.6

ml) and 0.3 to 2.8 ng/ml (mean 1.5 ng/ml), respectively. The plasma elimination half-life was in the range of 10.4–39.2 h (mean 18.4 h) for the parent compound and 11.2–45.0 h (mean 20.2 h) and 16.4–49.9 h (mean 27.9 h) for the metabolites II and IV.

Consistent with the elimination half-life, the parent compound and its metabolites slowly ac-

TABLE II

SUMMARY OF QUALITY CONTROL RESULTS FOR COMPOUND I AND ITS METABOLITES II AND IV IN HUMAN PLASMA FROM THE DAY-TO-DAY VALIDATION STUDY

Results are mean and S.D. (n = 8).

Concentration added (ng/ml)	Concentration found (ng/ml)	Coefficient of variation (%)	Accuracy (%)
Parent compound			
1.25	1.33 (0.10)	7.5	6.4
5	5.04 (0.34)	6.8	0.8
25	25.31 (0.92)	3.6	1.2
Metabolite II			
5	5.08 (0.43)	8.5	1.6
20	19.68 (1.47)	7.5	−1.6
100	102.15 (1.84)	1.8	2.2
Metabolite IV			
0.31	0.34 (0.02)	5.9	9.7
1.25	1.26 (0.06)	4.8	0.8
6.25	6.06 (0.14)	2.3	−3.0

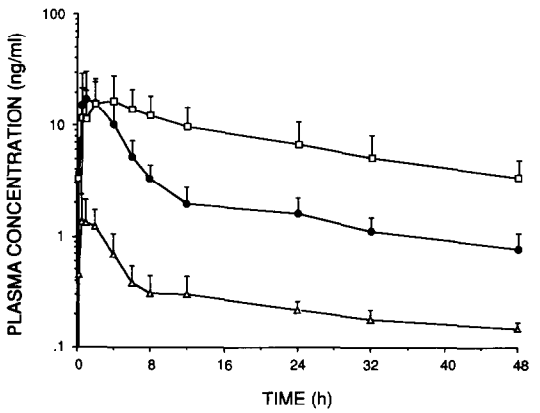

Fig. 5. Mean plasma concentration–time course of compound I (●) and its metabolites II (□) and IV (△) after oral administration of a 50-mg tablet of compound I to ten healthy adult male volunteers.

cumulated during chronic administration, achieving steady-state concentrations (C_{ss}) within four to seven days. Plasma C_{ss} values of metabolite II were, on average, four to five times those of compound I, although there was some inter-subject variability in the metabolite-to-parent drug ratio, as expected from the single-dose kinetic results (Table III shows the C_{ss} values in healthy subjects given daily 50- or 100-mg tablets of compound I for two weeks). Together with the fact this metabolite retains much of the parent compound's biological activity *in vivo* and *in vitro* (T. Mennini and R. Samanin, personal communication), this suggests that it may contribute to the pharmacological effects of compound I. Metabolite IV too has activity *in vitro* in some biological systems, but since it hardly reached 10% of the parent compound C_{ss} it would add little to the pharmacological action of compound I. As with centrally acting drugs in general [13], however, the contributions of each metabolite to the central activity will depend not only on their intrinsic activity but also on the extent to which they enter the brain compared with the parent compound.

TABLE III

STEADY-STATE PLASMA CONCENTRATIONS OF COMPOUND I AND ITS METABOLITES II AND IV IN HEALTHY MALE VOLUNTEERS

Compound I was administered daily for two weeks as tablets. Results are mean and S.D. (n = 5).

Dose (mg)	Steady-state plasma concentrations (ng/ml)		
	Compound I	Metabolite II	Metabolite III
50	8.8 (6.1.)	40.4 (21.0)	0.7 (0.3)
100	22.9 (5.2)	85.0 (15.6)	2.4 (0.7)

CONCLUSIONS

An HPLC procedure with post-column fluorescence photolysis has been developed and validated to measure the plasma concentrations of compound I and its metabolites II and IV that might be met in monitoring and pharmacokinetic studies. The method is selective and has detection limits of 1.25, 5 and 0.31 ng/ml for I, II and IV respectively, using 1 ml of plasma. The assay is linear in the range of concentrations tested, with mean C.V. and R.E. values generally below 10%. The method may be applied to assay rat and dog plasma in pharmacological and toxicological studies, achieving substantially better sensitivity than UV detection [8] and with some advantage over the post-column derivatization technique [11] (*i.e.* no mixing problems, less pump noise and no need for an extra pump). Moreover, it can be aplied to determine the brain concentrations of these compounds, after appropriate homogenization of the tissue (0.1 *M* hydrochloric acid, data not shown). Although the chromatographic run takes more than 30 min because four compounds are separated isocratically, a sample processor can be used to do the chromatographic analysis overnight.

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