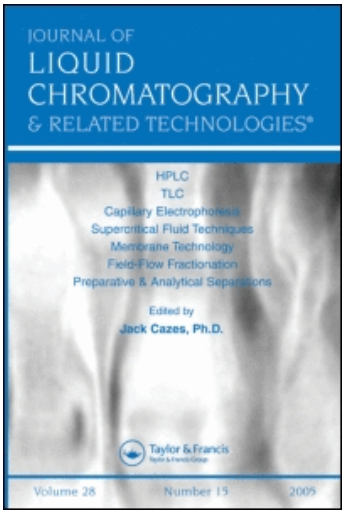

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G. Guiso[a]; S. Confaloneeri[a]; S. Gherardi[b]; M. Guido[b]; S. Caccia[a]
[a] Istituto di Ricerche Farmacologiche "Mario Negri" via Eritrea 62, Milan, Italy [b] Cyanamid Italia, Catania, Italy

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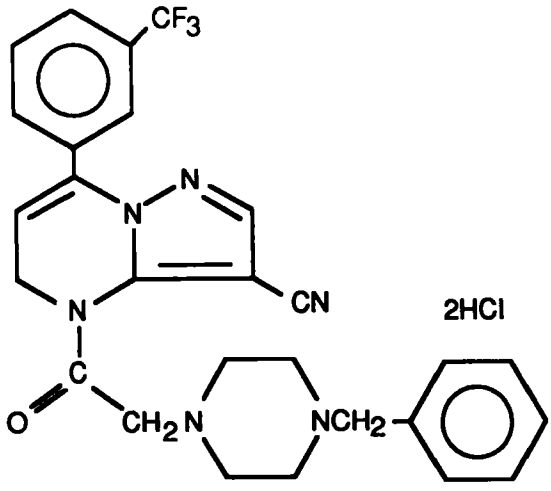
LIQUID CHROMATOGRAPHIC DETERMINATION OF THE POTENTIAL MEMORY ENHANCING AGENT CL 275,838 USING A POST-COLUMN PHOTOLYSIS AND FLUORIMETRIC DETECTION

G. GUISO[1], S. CONFALONIERI[1], S. GHERARDI[2], M. GUIDO[2], AND S. CACCIA[1]

[1]*Istituto di Ricerche Farmacologiche "Mario Negri"*
via Eritrea 62
20157 Milan, Italy
[2]*Cyanamid Italia*
95030 Catania, Italy

ABSTRACT

A method has been developed for quantitation of the potential memory-enhancing agent CL 275,838 (I) in plasma. After isolation of the compound I and the internal standard from plasma constituents using a Sep-Pak C_{18} cartridge and separation by reverse-phase liquid chromatography, the eluate peaks were passed through a PTFE photolysis coil irradiated by an UV lamp, resulting in conversion of the compounds to highly fluorescent species. The method was sensitive, precise and easily applicable for the automatic injection of series of plasma samples from healthy males in preliminary clinical studies of the oral compound I (10-100 mg). Absorption of I was rapid and peak plasma concentrations and area under the curve increased with the dose, suggesting that the pharmacokinetics of I are linear in the dose range investigated.



(I)

Figure 1: Chemical structure of CL 275,838, 4,5-dihydro-4-[[4-(phenylmethyl)-1-piperazinyl)acetyl]-7-[3-(trifluoromethyl)-phenyl]-pyrazolo[1,5,-a]pyrimidine-3-carbonitrile dihydrochloride

INTRODUCTION

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, is a potential memory-enhancing compound structurally (see Fig. 1) and pharmacologically (American Cyanamid, data on file) unrelated to any existing drug with similar indications (see 1-3 for reviews of this heterogeneous class of drugs). Clinical trials are under way to test the efficacy of I in humans and a validated analytical method for measuring it in body fluids is a prerequisite for such studies.

Initial disposition studies of the ^{14}C-labelled compound in animals showed it was well absorbed from the gastro-intestinal tract but extensively

distributed and metabolized resulting in low circulating concentrations of unchanged compound. In healthy human volunteers given single and multiple doses of 5 mg of CL 275,838, plasma concentrations of unchanged compound reached a maximum of 3-14 ng/ml. Therefore, a method for quantifying the compound in human plasma must have a nanogram per milliter detection limit to permit a useful working range. Such sensitivity could not be achieved using the high performance liquid chromatographic method (HPLC) with ultraviolet detection previously developed (4). However, using HPLC with post-column oxidation of the eluate and fluorescence detection, a sensitive alternative has been recently introduced for clinical kinetic studies of I (5).

The present report describes a modification of this fluorimetric technique which uses post-column photolysis instead of alkaline degradation to convert CL 275,838 and the internal standard to highly fluorescent species. This new procedure has some advantages (ie no mixing problems, less pump noise and no need for an extra pump) over the post-column derivatization techniques (5) and is easily applicable for the automatic injection of samples down to 1 ng/ml CL 275,838. An extensive review of the use of photochemical reactions for post column derivatization has recently appeared (6).

MATERIALS AND METHODS

Standard solution and solvents

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

Stock solutions of compound I and the I.S were prepared weekly in acetonitrile at a concentration of 1 mg ml^{-1}. Standard solutions (1 $\mu g\ ml^{-1}$) 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, C. Erba, Milan, Italy; phosphoric acid, E. Merck, Darmstadt, Germany) were of analytical-reagent grade and were used without further purification.

Sample preparation

Sep-Pak C_{18} cartridges (Waters Assoc., Milford, USA) were pre-wetted with 5 ml of acetonitrile, 5 ml of acetonitrile-water (50:50 v/v) and 5 ml of distilled water. Two ml of human plasma (containing 2.5 ng of I.S.) were then added and the cartridges were washed with 5 ml of distilled water, 5 ml of acetonitrile-water (20:80 v/v) and 0.35 ml acetonitrile. The compounds of interest were removed by eluting the cartridges with 2 ml of acetonitrile, then evaporated to dryness in vacuo. The residue was dissolved in 0.2 ml of the mobile phase (see below), centrifuged in Eppendorf tubes at 12,000 g for 5 minutes and the clean supernatant was analyzed by HPLC using post-column photolysis 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., Milford, MA, USA) were used. A reversed phase column (Supelco LC 18 DB, 15 cm x 4.6 mm), maintained at room temperature, and a Newguard RP18 precolumn (Brownlee Labs., Dupont, Santa Clara, USA) were fitted. The mobile phase was CH_3CN/CH_3OH/0.1M NaH_2PO_4/0.1 M H_3PO_4 (30:22:24:24 v/v) 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 admixed to a photochemical reaction unity "Beam Boost" (ICT-International Chromatography Technology-Gmbh, Frankfurt, Germany). Photoxidation was accomplished in a reaction coil of 0.3 mm I.D. and length 10 m (irradiation time 60 s), coiled around the UV lamp (254 nm). A Shimadzu RF 535 fluorescence monitor using an excitation wavelength of 335 nm, and a Corning CS3-73 Sharp-cut Filter (cut wavelength 416-436 nm) in the emission path were used. The detector was coupled to a C-R6A Chromatopac Shimadzu integrator (A.STR.AN, Shimadzu) for determination of peak height.

Validation

The precision and reproducibility of the method was determined by replicate analyses of quality control (QC) samples containing known concentrations of I (1.25-50 ng/ml). With each day's analysis, these QC

samples were assayed with standard samples, and the calculated concentrations were compared (inter-assay variance). Intra-assay variance was checked by replicate analysis of QC samples (four at each concentration) on the same days. Standard calibration curves were constructed daily 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 (I.S. method). Concentrations of I in the validation samples were determined by interpolation from the calibration curves using peak-height ratios from the samples.

Recovery was determined by comparing the peak heights of I after injection of non-extracted standard solutions and after injection of extracted plasma containing equal concentrations of the compound. The specificity of the assay was checked by running blank human plasma.

Application

Ten healthy male volunteers aged between 19 and 42 years and within 15% of normal body weight (62-86 kg) with normal clinical and biochemical profiles took part in the study. Each volunteer was screened for suitability for entry and was accepted only if the medical history was satisfactory and there was no evidence of diabetes, cardiovascular, hepatic, renal or gastrointestinal disease, mental disorders or depressive illness. 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. All subjects had been free from medication for at least one week before the study.

After an overnight fast, volunteers ingested either 10 or 50 mg (group A) or 20 or 100 mg (group B) I tablet (kindly supplied by Cyanamid, Catania, Italy) with water in two separate occasions. They were allowed only water for 4 h after dosing. A venous blood sample were drawn before (time 0) and nominally 0.5, 1, 2, 3, 4, (approximately around the peak plasma concentration) (5) and 24 h after dosing. Each sample was processed to produce plasma which was stored frozen (-20°C) until analysis.

The area under the plasma concentration-time curve (AUC) was calculated by the trapezoidal method (7). The maximum plasma concentration (C_{max}) and the time (t_{max}) of its occurrence were read directly from the plasma concentration-time data.

RESULTS AND DISCUSSION

On irradiation with short-wavelength ultraviolet (UV) light, the potential memory enhancing compound I is cleaved into a derivative that can be measured by its strong fluorescence with maximum emission at 416-436 nm. Based on the chromatographic retention time of the authentic compound kindly supplied by American Cyanamid we established that the photochemical reaction involves the formation of 7-[3-(trifluoromethyl) phenyl]-pyrazolo[1,5,-a]pyrimidine-3-carbonitrile (CL 228,346), a metabolite (IV) of I identified animal body fluids and tissues (American Cyanamid, data on file). The same reaction occurs under alkaline conditions; it probably involves hydrolysis of the acyl group of I at position 4 of the pyrazolpyrimidine moiety followed by oxidation of the product of hydrolysis (5).

A Sep-Pak C_{18} cartridge was used to clean up the plasma samples, this form of preparation being simple and fast. Recovery was 75-80% over the 1.25-50 ng/ml range and the chromatograms did not indicate any interfering endogenous peaks. Examples of chromatograms of a drug-free plasma (A), a drug-free plasma suppementated with I (1.25 ng/ml) (B) and plasma from a healthy volunteer given 5 mg of I (C) are shown in Fig. 2. Approximate retention times were 6 min for I and 16 min for the I.S.

A non-interfering peak corresponding in retention time to authentic CL 228,346 (metabolite IV) was observed in chromatograms from human volunteers given I orally. This is the only postulated metabolite that can be simultaneously measured (recovery 90 ± 5%, lower limit of detection 0.3 ng/ml) with the procedure described. However, this appears to be only a minor metabolite of I in man, never amounting to 15-20% of the parent drug plasma concentrations in terms of C_{max} and AUC (data not shown). Other putative metabolites of compound I have different extraction characteristics (5) or even exhibit no fluorescence on irradiation with short-wavelength UV light and therefore cannot be measured by the proposed method.

The graphic relationships between the peak-height ratios of I to I.S. and the amount of the compound added to plasma were always linear from 1.25 to at least 50 ng/ml. The coefficient of determination (r) for each standard curve invariably exceeded 0.99. The slopes of 5 curves, plotted over a period of three weeks, had a coefficient of variation (CV) of 2.3% (average regression equation: $y = 0.059x - 0.006$). The lower limit of quantitation was

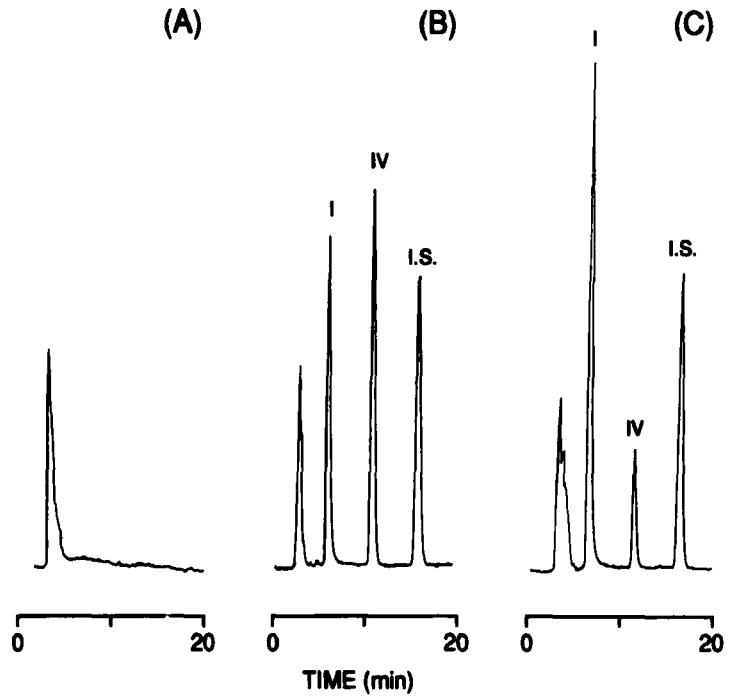


Figure 2: Chromatograms of extracts from (A) drug-free plasma, (B) drug-free plasma suppemented with compound I (10 ng/ml), its metabolite IV (2.5 ng/ml) and the internal standard (I.S., 2.5 ng/ml) and (C) plasma of a healthy volunteer taking 50 mg of I orally and containing 16.3 ng/ml of unchanged compound and 0.9 ng/ml of its metabolite IV.

approximately 1.25 ng/ml using 1 ml of plasma. This limit can be reduced still further using 2-3 ml of plasma, recovery and specificity both being relatively unaffected by these different volumes.

Human plasma QC samples containing the equivalent of 1.25-50 ng/ml of I were assayed with each of the HPLC chromatographic runs in support of this study. The results, summarized in Table 1, indicated a C.V. of intra-assay precision at the low levels of about 8% and in the range of 1.8-6.3 % for all higher concentrations. Inter-assay C.V. were 3.0-8.3%; the relative error of

TABLE 1

Precision and Reproducibility for Plasma Samples Spiked with Compound I

Amount added (ng/ml)	Amount found (ng/ml with SD)	Coefficient of variation (%)
Within-day (n = 4)		
1.25	1.3 (0.1)	7.7
2.5	2.5 (0.2)	8.0
5	4.8 (0.3)	6.3
10	10.1 (0.5)	5.0
25	24.9 (0.8)	3.2
50	49.7 (0.9)	1.8
Day-to-day (n = 12)		
2.5	2.4 (0.2)	8.3
10	9.8 (0.5)	5.1
50	49.4 (1.5)	3.0

these QC samples R.E.= [(F-A)/A X 100], calculated from the deviation of the concentration found (F) from the nominal value (A), indicated an interassay variation from -1.2 to -4%. Thus the precision and accuracy of the method appear acceptable over the concentration range investigated.

An example of the potential use of the analytical procedure is given in Fig. 3, which shows the relationship between the dose of I and mean plasma C_{max} and AUC calculated up to 4h in healthy male volunteers. The t_{max} ranged from 0.25 to 2 h regardless of the dose. Mean C_{max} and AUC (with SD) increased from 6 (3) ng/ml and 13.8 ng/ml . h at 10 mg to 50.2 (23.4) ng/ml and 104.2 (32.5) ng/ml . h at the 100 mg dose. Least-squares linear regression of the single values (n = 5) against the dose gave an overall correlation coefficient of 0.75 ($P < 0.01$) for C_{max} and 0.78 ($p < 0.01$) for AUC. Thus, there was a significant linear relationship between oral bioavailability (eg, C_{max} and AUC_{0-4h}) and dose in healthy males.

CONCLUSIONS

An HPLC procedure with post-column fluorescence photolysis has been developed and validated to measure the plasma concentrations of CL

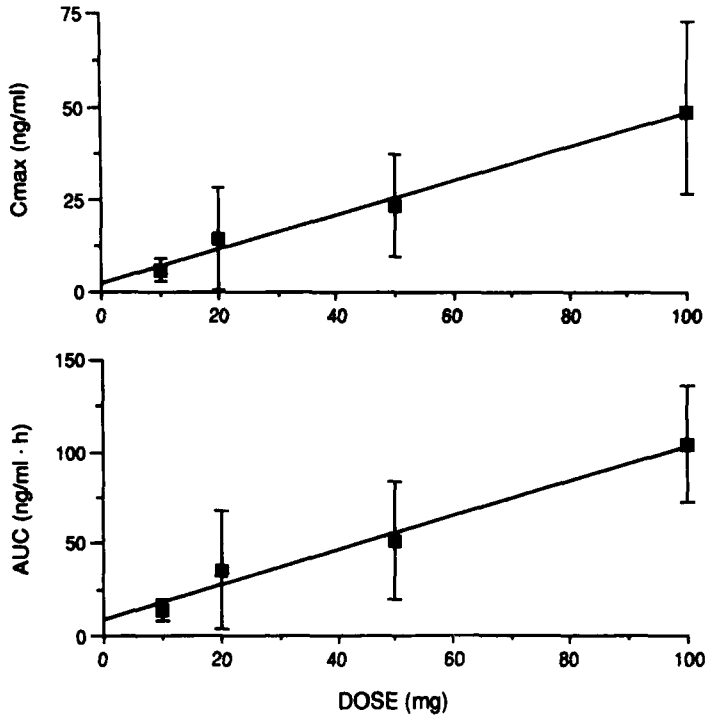


Figure 3: Relationship between compound I dose and maximum plasma concentration (C_{max}, $r = 0.75$, $p < 0.01$) and area under the curve ($AUC_{0\text{-}4h}$, $r = 0.78$, $p < 0.01$). Each point is the mean (± SD) of five volunteers.

275,838 that might be met in pharmacokinetic and monitoring studies. The method is sensitive, precise and linear in the range of concentrations likely to be present in plasma of volunteers after possible therapeutic doses.

Initial kinetic studies of I in healthy male volunteers indicate that absorption of the compound is rapid but oral bioavailability varies widely between subjects. Such inter-subject variability is common to many centrally-acting lipophilic agents and is consistent with the concept of an extensive first-pass metabolism of high-extraction drugs (8, 9). However, mean C_{max} and

AUC_{0-4h} of I in healthy volunteers increased proportionally with the dose, suggesting that first-pass metabolism is not saturated by single doses in the 10 to 100 mg range.

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