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Note

High-performance liquid chromatographic assay for a novel psychotropic drug (CI-943) in cynomolgus monkey plasma

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8-Ethyl-7,8-dihydro-1,3,5-trimethyl-1*H*-imidazo [1,2-*c*]pyrazolo[3,4-*e*]-pyrimidine (CI-943, I, Fig. 1) is a potential agent for the treatment of psychotic disorders. I has been shown to produce antipsychotic-like effects in behavioral tests in rodents and monkeys [1–3]. Preclinical results in rats and monkeys suggest that I is not a brain dopamine antagonist and should have reduced liability in causing extrapyramidal syndrome at effective dose levels [1,2]. The molecular mechanism of action has not been elucidated [4]. Studies in experimental animals demonstrate that I accelerates brain dopamine turnover, al-

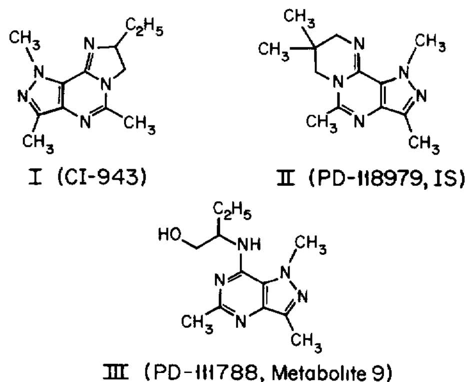

Fig 1 Structures of I, II (internal standard) and III

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though it does not share affinity for many neurotransmitter receptors as do traditional antipsychotic agents. Thus, I may offer a novel mechanism of action involving indirect alterations in dopamine transmission [2,5]

This paper describes a sensitive method for the determination of I in cynomolgus monkey plasma by a solid–liquid extraction process and high-performance liquid chromatography (HPLC) with ultraviolet detection. One of our goals in developing this method was to chromatographically separate I from a closely eluting metabolite (metabolite 9, III, Fig. 1) of similar structure III has been identified as metabolite 9 in monkeys dosed with I The minimum detectable limit is 0.05 μg/ml at a signal-to-noise ratio of 4, requiring a sample volume of 100 μl.

EXPERIMENTAL

Reagents and materials

I (Lot W), the internal standard (PD 118979, II) (Lot R) and metabolite 9 (PD 111788, III) (Lot P) were synthesized in house at the Parke-Davis Pharmaceutical Research Division, Warner-Lambert (Ann Arbor, MI, U.S A.) (Fig. 1). Triethylamine (AR) was obtained from Aldrich (Milwaukee, WI, U.S.A.) 1-Dodecanesulfonate sodium was obtained from Regis (Morton Grove, IL, U.S.A.) and 1-heptanesulfonic acid sodium salt was purchased from Eastman Kodak (Rochester, NY, U S.A.). Sodium acetate (AR), orthophosphoric acid (AR), acetonitrile and methanol (Chrom-AR) were all obtained from Mallinckrodt (Paris, KY, U.S.A.). Solid-phase cartridges [Bond-Elut C_{18} cartridge, 3 ml (200 mg) capacity] were purchased from Analytichem International (Harbor City, CA, U.S.A.). Water, which was of HPLC grade, and sulfuric acid (AR) were obtained from Fisher Scientific (Fairlawn, NJ, U S A) Heparinized cynomolgus monkey plasma from Lampire Biologicals (Pipersville, PA, U.S.A.) was used.

Nine calibration standards were prepared daily over a concentration range of 0 05–3 0 μg/ml using a plasma sample size of 100 μl The internal standard was utilized at a concentration of 1.0 μg/ml and a volume of 100 μl per sample.

Cynomolgus monkey plasma quality control standards of 0.25, 1.5, and 2 5 μg/ml I were prepared by diluting aliquots of a 100 μg/ml I solution with drug-free monkey plasma to a final volume of 10 ml. Aliquots of quality control standards were stored frozen at −20°C.

Chromatography

A Waters (Milford, MA, U.S.A.) Model M-45 solvent delivery system, a Model 002027 TCM column heater and a Model 481 Lambda-Max LC spectrophotometer with ultraviolet detection at 315 nm were used in conjunction with a Hitachi (Tokyo, Japan) Model 655A-40 autosampler and a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3390A computing integrator. Mobile

phase was pumped at 0.7 ml/min. Chromatographic separation was achieved on two reversed-phase C_{18} Hypersil columns (Hewlett-Packard) placed in series (5 μm particle size, 100 mm×4.6 mm I D., followed by a 3 μm particle size, 60 mm×4 6 mm I.D.). An RP-8 Spheri-5 MPLC guard cartridge (5 μm particle size, 3 cm×4.6 mm I.D.) from Brownlee Labs. (Santa Clara, CA, U.S A) was used. Guard and analytical columns were maintained at 45°C.

The isocratic mobile phase consisted of acetonitrile–buffer (26 74, v/v). The buffer contained 0.004 *M* triethylamine, 0.0036 *M* sodium acetate and 0.0001 *M* dodecanesulfonate sodium The final solution was adjusted to pH 5.0 with concentrated orthophosphoric acid and filtered through a 0 45-μm nylon filter from Schott-Duran (Gottingen, F.R.G.).

Assay procedure

An aliquot of monkey plasma (either 100 μl of control plasma and 50 μl of an aqueous I standard or 100 μl of sample plasma and an equal volume of HPLC-grade water) and II solution (100 μl) were added together with 0 5 ml of an ion-pair solution [5 mg of 1-heptanesulfonate sodium salt in 500 ml of 0.5% (v/v) acetic acid] to a 75 mm×12 mm disposable glass culture tube. The tubes were mixed and allowed to remain at room temperature (22–27°C) during column preconditioning.

Solid-phase columns were preconditioned using five 1-ml volumes of methanol followed by five 1-ml volumes of HPLC-grade water. The plasma solution was passed through the cartridge using a gentle vacuum of 23 7 MPa. Each cartridge was rinsed sequentially with the following three rinses· 1 ml of 0.5% (v/v) acetic acid, 0.5 ml of 0 05 *M* sodium acetate and two 100-μl rinses of acetonitrile–0.025 *M* sodium acetate (20 80, v/v), with 1 μl triethylamine per ml of solution I and II were eluted from the cartridge into a clean collecting tube using acetonitrile–0.025 *M* sodium acetate (50 50, v/v), with 2 μl of triethylamine per ml of solution. Three 100-μl aliquots of the elution solution were passed through each cartridge for a total elution volume of 300 μl. Samples were evaporated to dryness in a 40°C water bath under a gentle stream of nitrogen A volume of 50 μl of methanol, 225 μl of HPLC-grade water and 25 μl of a 0.5 *M* sodium acetate solution (pH 4.0 with sulfuric acid) were used to reconstitute each sample, followed by mixing An aliquot of 275 μl was injected into the chromatographic system. All samples and standards were randomized prior to injection. To obtain the concentration of I in an unknown sample, the I/II peak-area ratio (peak area of I/peak area of II) of the nine extracted standards was used to construct a standard curve; the concentration of I was calculated by using the calibration equation obtained using weighted (weight= 1/concentration^2) least-squares linear regression analysis [6,7].

Validation procedure

System precision and linearity of I in monkey plasma was validated over the concentration range 0.05–3.0 μg/ml by analyzing nine calibration standards of

I in triplicate on three separate days. Accuracy of the assay was determined by analyzing quality control samples at three concentrations in triplicate on each of the three days Percent recovery of I and II was determined at three concentrations and one concentration, respectively.

RESULTS AND DISCUSSION

Chromatography and selectivity

Fig. 2A and B illustrate two chromatograms from a single cynomolgus monkey: one from a pre-dose sample and the other from a 1-h post-dose sample following oral dosing with I at 50 mg/kg body weight. The concentration of I

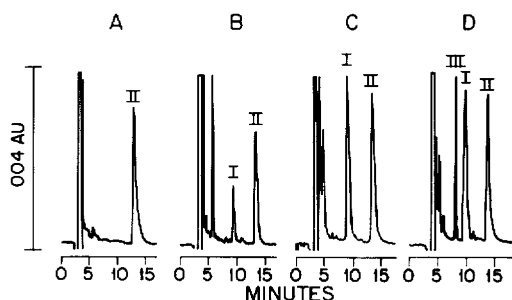

Fig 2 Chromatograms of (A) extracted plasma with internal standard (II) from a pre-dose monkey sample, (B) extracted plasma with internal standard (II) from the same monkey 1 h following a 50 mg/kg oral dose of I, (C) extracted monkey plasma calibration standard containing 1 0 μg/ml I and II and (D) extracted monkey plasma calibration standard containing 1 0 μg/ml I, II, and spiked with 0 5 μg/ml III

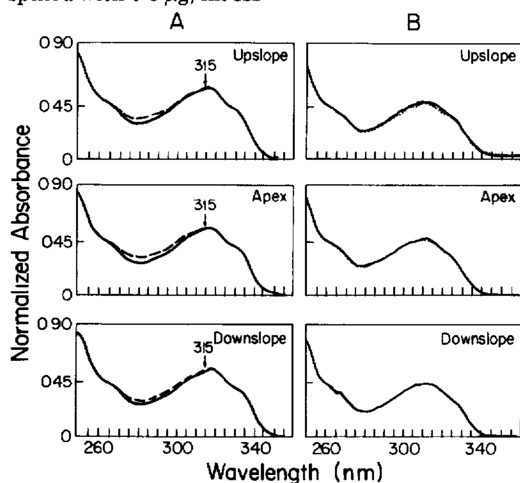

Fig 3 UV photo-diode array plots of (A) I and (B) II at upslope, apex and downslope from aqueous standard (—), monkey plasma calibration standard ( ) and monkey plasma sample (- -)

was 0 37 μg/ml The retention time of I was 9 8 mın and that of the ınternal standard 14 1 min under the condıtıons described Fıg 2C represents a 1 0 μg/ml monkey plasma calıbratıon standard with ınternal standard (II) Separatıon of I from metabolite 9 (III), a closely elutıng compound of sımılar structure found ın monkeys dosed wıth I, was achieved usıng thıs method (Fıgs 1 and 2D).

System selectıvıty was confirmed by multıple-wavelength rapıd-scan photodıode array detectıon of the upslope, apex and downslope of the chromatographıc peaks of I and II (Fıg 3) No extraneous peaks from extracted monkey samples ınterfered wıth the quantıtatıon of I or II at 315 nm

Precısıon and accuracy of the calıbratıon curve

System precısıon and linearity were valıdated ın cynomolgus monkey plasma by analyzıng nıne calıbratıon standards contaınıng 0.05–3 0 μg/ml I ın trıplıcate over a three-day perıod Reproducibility of peak-area ratıos over the calıbratıon range, expressed as coefficıent of varıation (C V), ranged from 2 5 to 13 0% Precısıon of calıbratıon standards, expressed as C V., ranged from 2 8 to 10 4% wıth a relatıve error (R E.) of wıthın ±5.3% (Table I)

Precısıon and accuracy of the method

Assay precısıon and accuracy were determıned by analyzıng three qualıty control pools ın trıplıcate on three separate days Assay precısıon was ±8 4% based on C V values of 8.4, 4.7 and 3 5% for controls contaınıng 0 25, 1 5 and 2 5 μg/ml I, respectıvely. The accuracy of the method was ±2 8%, wıth R E. values of 2.0, 0 67 and 2 8% for the same three control pools (Table II)

TABLE I

MEAN PEAK-AREA RATIOS AND CALCULATED CONCENTRATIONS OF CALIBRATION STANDARDS OF I IN CYNOMOLGUS MONKEY PLASMA ($n=9$)

Concentratıon added (μg/ml)	Mean peak-area ratıo	C V (%)	Mean concentratıon found (μg/ml)	C V (%)	Relatıve error (%)
0 05	0 044	13 0	0 050	10 4	0 0
0 075	0 075	10 7	0 079	8 0	5 3
0 125	0 116	7 4	0 119	4 7	−4 8
0 25	0 249	6 1	0 247	5 0	−1 2
0 5	0 521	2 9	0 510	3 8	2 0
1 0	1 00	2 5	0 976	3 1	−2 4
1 5	1 56	4 8	1 52	5 2	1 3
2 5	2 67	2 5	2 60	2 8	4 0
3 0	3 05	5 2	2 95	3 8	−1 7

TABLE II

MEAN CONCENTRATIONS OF QUALITY CONTROLS OF I IN CYNOMOLGUS MONKEY PLASMA

Concentration added (μg/ml)	n	Mean concentration found (μg/ml)	C V (%)	Relative error (%)
0 25	9	0 245	8 4	−2 0
1 5	9	1 51	4 7	0 67
2 5	8	2 57	3 5	2 8

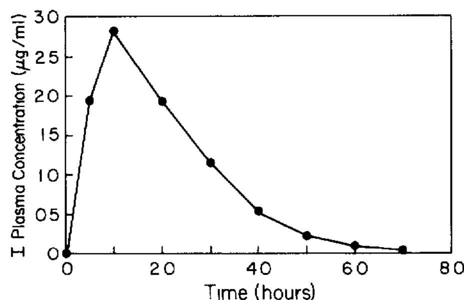

Fig 4 Plasma concentration versus time profile obtained following oral administration of 20 mg/kg I to a cynomolgus monkey

Plasma recovery

The recovery of I from cynomolgus plasma was determined at 0.125, 0 25 and 2 5 μg/ml. The mean recovery of eight or nine samples at each concentration was 73, 71 and 75% with C.V. values of 12 9, 8 6 and 10.0%, respectively The recovery of II at a concentration of 1.0 μg/ml from 27 standards was 68% with a C V of 8 2%

Animals and drug administration

Plasma samples obtained from cynomolgus monkeys following oral administration of I were processed as described. A plasma concentration versus time curve for a cynomolgus monkey administered an oral dose of 20 mg/kg I is illustrated in Fig. 4.

CONCLUSION

A precise, accurate, selective and sensitive liquid chromatographic method for the determination of I in monkey plasma has been developed. More than 600 monkey plasma samples from toxicological support and pharmacokinetic studies have been processed using this method. Separation of I from a potential interfering metabolite (III) has been achieved The method is linear over the

concentration range of 0.05–3.0 μg/ml and the detection limit is 0.05 μg/ml at a signal-to-noise ratio of 4.

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