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Determination of a novel α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor antagonist (LY300164) and its N-acetyl metabolite in mouse, rat, dog, and monkey plasma using high-performance liquid chromatography with ultraviolet detection

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Abstract

A method for the analysis of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptor antagonist LY300164 (compound I) and its N-acetyl metabolite (compound II) in plasma was developed. The assay utilized solid-phase extraction on a C_{18} Bond Elut cartridge followed by reversed-phase HPLC with UV detection at 310 nm. The method exhibited a large linear range from 0.05 μg/ml to 50 μg/ml with an intra-assay accuracy for compound I and compound II ranging from 89.0% to 114.5% and intra-assay precision ranging from 0.5 to 15.3% in mouse, rat, dog, and monkey plasma. The inter-assay accuracy of compound I and compound II was 93.3% to 101.8% and the inter-assay precision was 1.6% to 11.2% in dog plasma. The lower limit of quantitation was 0.05 μg/ml for compound I in plasma from all species tested. The lower limit of quantitation for compound II was 0.05 μg/ml in dog and monkey plasma and 0.1 μg/ml in mouse and rat plasma. Extracts of compound I and II from dog plasma were shown to be stable for 24 h at room temperature, and both compounds were stable when spiked into rat and monkey plasma frozen at −70°C for 27 days. The method has shown to be useful in the investigation of the pharmacokinetics of the parent compound (I) and metabolite (II) in preclinical studies.

1. Introduction

Excitatory amino acids are thought to play a major role in synaptic transmission in the mammalian central nervous system [1] and have been implicated to play a role in acute and possibly chronic neurodegenerative diseases such as Parkinson's disease [2], Amyotrophic Lateral Sclerosis [3,4], epilepsy [5–7] and neurodegeneration involved in stroke [8]. In recent years, a number of 2,3-benzodiazepines have been found to act as excitatory amino acid AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptor antagonist with no potentiation of the γ-aminobutyric acid (GABA) receptor inhibition found in classical 1,4-benzodiazepines [9–11]. The compound LY300164, (*R*)-7-acetyl-5-(4-aminophenyl)-8,9-dihydro-8-methyl-7H-1,3-dioxolo[4,5-*h*]benzodiazepine referred to as compound I, is a selective noncompetitive antagonist

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of the excitatory amino acid AMPA receptor which has shown efficacy as an anticonvulsant in animal models and is currently in preclinical development for the treatment of epilepsy. Initial studies in mice, rats, dogs, and monkeys indicated that the rate of N-acetylation of compound I was variable among species. Development of an analytical method to detect and quantitate both compound I and II in mouse, rat, dog, and monkey plasma was therefore important to preclinical pharmacokinetic studies. Initial experiments showed that LY300164 was easily extracted from plasma by reversed-phase solid-phase cartridges and could be detected with UV at 310 nm.

2. Experimental

2.1. Reagents and materials

The internal standard which is the propyl derivative of compound I, and compound II were synthesized at Eli Lilly, Indianapolis, IN, USA. Compound (I) was synthesized at Eli Lilly, Erl Wood, UK. The potency for compound I was determined to be 95.21%. See Fig. 1 for structures of compound I, compound II, and internal standard. All deionized water used in HPLC buffers was purified with a Waters MilliQ purification system. HPLC-grade methanol and acetonitrile were obtained from Burdick and Jackson. All other reagents were of analytical reagent grade. Heparinized mouse and rat plasma were obtained from Harlan Bioproducts (Indianapolis, IN, USA). Heparinized dog and monkey plasma was obtained from stock animals at Eli Lilly Toxicology facilities (Greenfield, IN, USA).

2.2. Apparatus and conditions

A Varian 9010 pump equipped with a Varian 9095 autosampler and Varian 9050 UV detector was utilized in all analytical procedures. Samples were extracted using a Supelco vacuum manifold. All glassware was silylated in a vacuum oven at 150°C using hexamethyldisilazane.

2.3. Preparation of stock solutions

A single stock solution was prepared for compounds I and II at 1.0 mg/ml in 50% acetonitrile. Dilutions of the initial stock solutions were made in 50% acetonitrile to give working standard solutions of 100 μg/ml, 10 μg/ml, and 1.0 μg/ml used for preparation of spiked plasma samples and all standard curve samples. Internal standard stock solutions were prepared at 10 μg/ml in 50% acetonitrile. All stock solutions were prepared in amber vials each day of analysis.

2.4. Sample preparation

The concentrations of compound I and II were determined from 200 μl of plasma. A total of 50 μl of internal standard stock (10 μg/ml) was

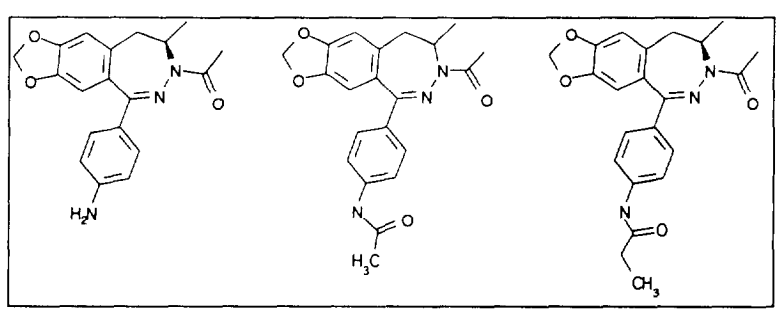

Fig. 1. Structures of compound I, compound II (metabolite), and the internal standard (I.S.).

added to each sample followed by 0.5 ml of deionized water. All standard and spiked samples were treated in an identical fashion. Standards were prepared at 0.05, 0.1, 1.0, 5.0, 20, and 50 μg/ml of compound I and II in mouse, rat, dog or monkey plasma. Control plasma samples were spiked at 0.05, 0.1, 1.0, and 50.0 μg/ml of compound I and II in dog plasma, 0.050, 5.0, and 50.0 μg/ml in monkey plasma, and 0.05, 0.10, 5.0 and 50.0 μg/ml in mouse and rat plasma. Solid-phase C 18 extraction cartridges (Bond Elute No. 1210–2001) were conditioned with 4.0 ml of methanol followed by 4.0 ml of MilliQ water. Plasma samples were extracted with a Supelco manifold under a vacuum of approximately 20 mmHg. Each sample was washed with 2.0 ml of MilliQ water followed by 2.0 ml of 10% methanol in water. Compounds I, II, and the internal standard were then eluted from the cartridges into a 13 × 100 mm glass tube using 400 μl of methanol. Each sample was concentrated to dryness at 80°C under a stream of nitrogen, redissolved in 200 μl of 35% methanol in water and mixed by vortexing. Samples were transferred to a 200-μl HPLC vial insert in a 2.0-ml vial. A total of 75 μl was injected onto a YMC basic 15 cm × 4.6 mm I.D. reversed-phase column (YMC Co. No. B-02-05) with a flow-rate of 1.0 ml/min. The mobile phase consisted of methanol–0.067 *M* citrate buffer pH 5.0–acetonitrile in a premixed solvent ratio of 40:50:10. All compounds of interest were detected by UV at 310 nm. Chromatographic analysis were performed with a Perkin Elmer Nelson Access Chromatography System.

The quantitation of compounds I and II was determined on three separate days in dog plasma at concentrations ranging from 0.05 to 50 μg/ml. Concentrations of compound I and II were determined in monkey, mouse, and rat plasma over the same concentration ranges for one day. The accuracy and precision was calculated to define limits of quantitation for all species.

2.5. Extraction efficiency and stability

The extraction efficiency for compounds I and II was determined in dog plasma at concentrations of 0.05 μg/ml, 5.0 μg/ml, and 50.0 μg/ml. Extraction efficiency was calculated by dividing the peak area of each compound at the three concentrations following extraction from dog plasma by the peak area of compound I and II estimated from reagent standards prepared at identical concentrations without extraction. The stability of compound I, II, and the internal standard in dog plasma extracts was investigated by comparing peak areas of samples reinjected at approximately 24 h after extraction to the peak area of the same samples following initial analysis. (Stability in this case was percentage obtained by dividing the peak area of each compound at 24 h by the peak area at initial analysis). Rat and monkey plasma spiked with compounds I and II at 5 μg/ml was analyzed by the method described herein immediately after storage at −70°C for 7, 17, and 27 days in order to measure the stability of both compounds at −70°C.

2.6. Animal study

Adult male Fischer rats were dosed with 20 mg/kg of compound I by oral gavage. A total of 45 rats were dosed ($n = 3$ animals per time point) and blood was collected at 0.083, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 16, and 24 h post-dose by cardiac puncture. All samples were centrifuged at 2500 rpm for 10 min and plasma was transferred to plastic polypropylene vials and stored at −70°C. All plasma samples were analyzed by the procedure described for compound I and II.

3. Results and discussion

Fig. 1 shows the structures of compound I, II, and the internal standard used in this assay. Reversed-phase chromatography of the compounds on a YMC basic column under the assay conditions yielded retention times of 5.3, 6.5, and 9.1 min for compound I, II, and the internal standard, respectively. The method was selective with no significant interferences at the retention times for compound I, II, or internal standard. Typical chromatograms of extracted blank dog

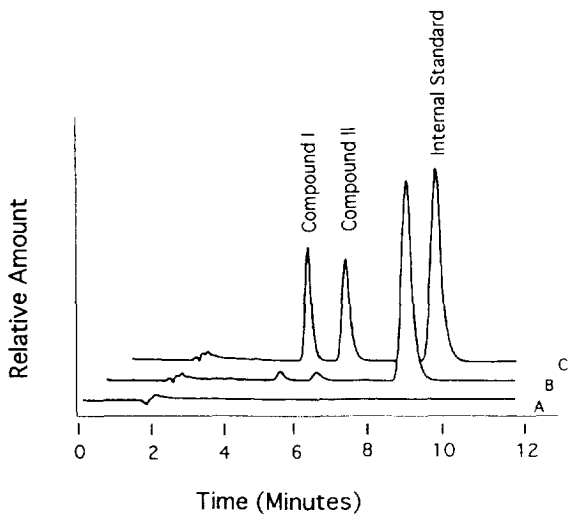

Fig. 2. Representative chromatograms of compound I, compound II (metabolite) and the internal standard used in the analytical procedure. (A) Blank dog plasma, (B) dog plasma spiked with 0.05 μg/ml of compound I and II, (C) dog plasma spiked with 1.0 μg/ml of compound I and II.

plasma and plasma spiked with 0.05 and 1.0 μg/ml of compound I and II are shown in Fig. 2. The standard curves were linear over the entire range of 0.05 μg/ml to 50.0 μg/ml for compound I and II with correlation coefficients (r^2) greater than 0.99. The assay precision and accuracy were determined in dog plasma over 3 days at plasma concentrations of 0.05, 0.1, 1.0, and 50 μg/ml for compound I and II. The three day validation in dog plasma yielded an intra-assay accuracy for compound I ranging from 89.0% to 106.4% and intra-assay precision ranging from 0.7% to 14.2% (Table 1). The inter-assay accuracy ranged from 93.3% to 101.4% and the inter-assay precision ranged from 1.7% to 11.2%. For compound II, the intra-assay accuracy ranged from 91.5% to 105.6% and the precision from 0.8% to 13.6% (Table 2). The inter-assay accuracy ranged from 93.7% to 101.8% and the inter-assay precision ranged from 1.6% to 7.8%. The lower and upper limits of quantitation for compound I and II in dog plasma were defined as 0.05 μg/ml and 50.0 μg/ml.

The accuracy and precision of the assay were

Table 1
Calculated plasma concentrations of compound I for three days of analysis following extraction from dog plasma spiked with 0.05 to 50 μg/ml of compound I ($n = 5$)

Theoretical amount (μg/ml)	0.050	0.100	1.00	50.0
Day 1				
Average	0.047	0.097	0.89	50.7
Standard deviation	0.007	0.003	0.13	0.3
Accuracy (%)	94.5	97.1	89.0	101.4
Precision (C.V.)	14.0	3.2	14.2	0.7
Day 2				
Average	0.052	0.098	0.93	48.8
Standard deviation	0.003	0.004	0.10	0.4
Accuracy (%)	103.2	97.6	92.6	97.5
Precision (C.V.)	6.5	4.3	11.2	0.8
Day 3				
Average	0.053	0.102	0.98	52.6
Standard deviation	0.007	0.010	0.04	1.9
Accuracy (%)	106.4	101.8	98.4	105.1
Precision (C.V.)	13.2	10.0	3.9	3.5
Inter-assay average	0.051	0.099	0.93	50.7
Inter-assay accuracy	101.4	98.8	93.3	101.3
Inter-assay precision	11.2	5.8	9.8	1.7

measured on a single day in monkey, mouse and rat plasma and results are shown in Tables 3 and 4. The assay accuracy for compound I ranged from 97.9% to 114.5% and the assay precision ranged from 0.5% to 15.3% for the three species. The assay accuracy for compound II ranged from 97.2% to 107.0% and the precision ranged from 0.80% to 5.6% in monkey, mouse, and rat plasma. The lower and upper limits of quantitation for compound I in monkey, mouse, and rat plasma was 0.05 and 50.0 μg/ml. The lower limit of quantitation for compound II in monkey was 0.05 μg/ml, however, quantitation of compound II in rodent plasma at 0.05 μg/ml yielded accuracies of only 37% and 68.1%. The lower limit of quantitation in mouse and rat plasma was therefore set at 0.1 μg/ml.

The extraction efficiency was determined in dog plasma for both compound I and compound II at concentrations of 0.05 μg/ml, 5.0 μg/ml and 50.0 μg/ml ($n = 4$). The extraction efficiency for compound I was 99.3%, 86.2%, and 86.4%

Table 2
Calculated plasma concentrations of compound II for three days of analysis following extraction from dog plasma spiked with 0.05 to 50.0 μg/ml of compound II ($n = 5$)

Theoretical amount (μg/ml)	0.050	0.100	1.00	50.0
Day 1				
Average	0.050	0.097	0.92	49.5
Standard deviation	0.002	0.003	0.12	0.4
Accuracy (%)	100.9	96.9	91.51	98.9
Precision (C.V.)	4.7	3.5	13.63	0.8
Day 2				
Average	0.049	0.096	0.98	48.2
Standard deviation	0.006	0.007	0.01	0.4
Accuracy (%)	98.9	96.2	97.50	96.4
Precision (C.V.)	11.9	6.8	1.32	0.9
Day 3				
Average	0.053	0.098	0.92	47.7
Standard deviation	0.004	0.006	0.02	1.5
Accuracy (%)	105.5	97.6	92.2	95.5
Precision (C.V.)	6.8	6.4	2.18	3.0
Inter-assay average	0.051	0.097	0.94	48.5
Inter-assay accuracy	101.8	96.9	93.7	96.9
Inter-assay precision	7.8	5.6	5.7	1.6

Table 3
Calculated plasma concentrations of compound I following extraction from monkey, mouse, and rat plasma ($n = 4$)

Theoretical amount (μg/ml)	0.050	5.00	50.0
Monkey			
Average	0.057	4.96	50.1
Standard deviation	0.003	0.02	1.0
Accuracy (%)	114.5	99.3	100.3
Precision (C.V.)	5.4	0.5	1.9
Mouse			
Average	0.055	5.03	54.0
Standard deviation	0.008	0.30	0.6
Accuracy (%)	109.3	100.5	107.9
Precision (C.V.)	15.3	6.0	1.1
Rat			
Average	0.052	4.89	50.7
Standard deviation	0.002	0.04	0.4
Accuracy (%)	103.7	97.9	101.4
Precision (C.V.)	2.9	0.9	0.8

Table 4
Calculated plasma concentrations of compound II following extraction from monkey, mouse, and rat plasma ($n = 4$)

Theoretical amount (μg/ml)	0.050	0.100	5.00	50.0
Monkey				
Average	0.052	–	4.86	49.0
Standard deviation	0.003	–	0.04	1.0
Accuracy (%)	104.8	–	97.2	98.1
Precision (C.V.)	5.6	–	0.8	1.9
Mouse				
Average	0.019	0.103	5.09	52.7
Standard deviation	0.004	0.005	0.05	0.6
Accuracy (%)	37.0	103.0	101.9	105.4
Precision (C.V.)	23.7	4.4	1.0	1.1
Rat				
Average	0.034	0.107	4.90	50.4
Standard deviation	0.003	0.002	0.05	0.3
Accuracy (%)	68.1	107.0	98.0	100.8
Precision (C.V.)	7.7	2.0	1.0	0.5

at concentrations of 0.05, 5.0, and 50.0 μg/ml, respectively. For compound II, the extraction efficiency was 83.0%, 86.2% and 85.8% over the same concentration ranges. The standard deviation for determination of extraction efficiency was below 11.0 for both compound I and metabolite.

The stability of compound I, the metabolite and the internal standard was tested at 5.0 and 50.0 μg/ml in dog plasma extracts held at room temperature for 24 h ($n = 5$). There was no notable change in the peak area of these extracted samples at 24 h following extraction (peak areas at 24 h were 103.1% to 116.4% of the peak areas immediately following extraction). These data indicate that each compound is stable in dog plasma extracts for 24 h at room temperature. The stability of compound I and metabolite was examined in rat and monkey plasma stored at −70°C for up to 27 days. Results are shown in Fig. 3. On all days, the values for both compound I and its metabolite were within 8% of the concentrations determined immediately after spiking the plasma (day 0), suggesting both compounds were stable under the storage conditions investigated.

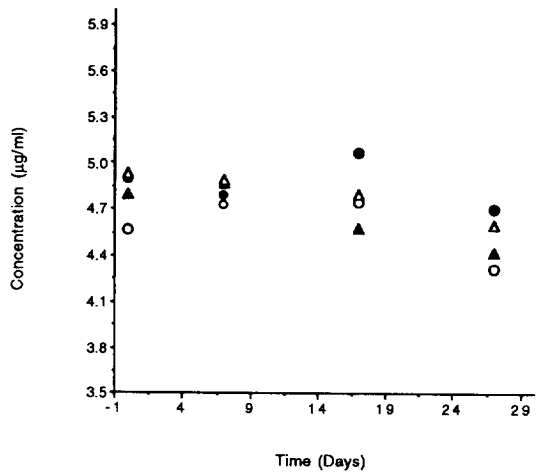

Fig. 3. Stability of compound I and compound II in plasma frozen at −70°C for 27 days ($n = 2$ for each data point). ○ = Rat plasma (compound I); ▲ = rat plasma (compound II, N-acetyl metabolite); ● = monkey plasma (compound I); △ = monkey plasma (compound II, N-acetyl metabolite).

Fig. 4 represents the plasma concentration of compound I and metabolite following oral administration of compound I to Fischer rats. The analytical method was sufficiently sensitive and selective to measure parent and metabolite up to 8 and 10 h post dose, respectively. The method is currently being utilized in preclinical drug metabolism studies in rats, mice, and monkeys following oral administration of compound I.

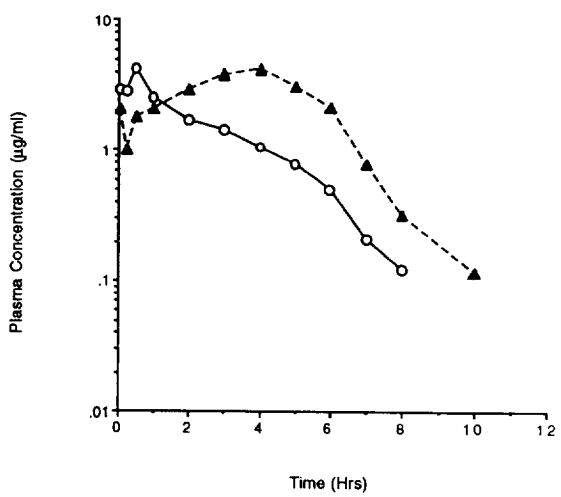

Fig. 4. Plasma concentrations of compound I (○) and the compound II (N-acetyl metabolite, ▲) determined by HPLC following oral administration of 20 mg/kg of compound I in the Fischer rat.

4. Conclusions

In conclusion, the method described extracted the AMPA receptor antagonist (compound I) and its metabolite successfully from plasma with excellent efficiency. The chromatography by reversed-phase was selective and rugged with sensitivity that allowed determination of preclinical pharmacokinetics of compound I and its metabolite following both oral and intravenous administration in mice, rats, dogs, and monkeys. Both compounds were stable for 24 h following extraction from plasma and 27 days in plasma stored at −70°C.

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