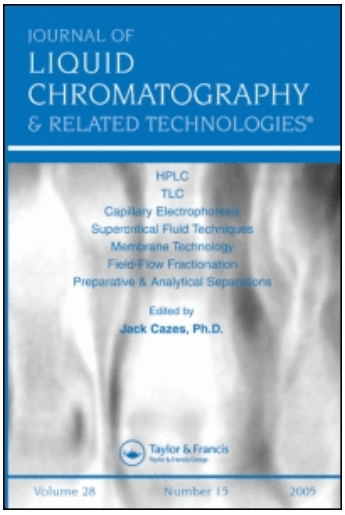

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Solid-Phase Extraction Followed by Purification with Tri-n-Butylphosphate as a Simple and Useful Rat Plasma Clean-up Method for HPLC-UV Determination of Zonampanel Monohydrate (YM872), a Highly Water-Soluble α-Amino-3-hydroxy-5-methylisoxazole-4-propionic Acid Receptor Antagonist

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Abstract: A high-performance liquid chromatographic method with ultraviolet detection was developed for the determination of zonampanel monohydrate (YM872), a novel α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor antagonist, in rat plasma. YM872 was extracted via solid-phase extraction. To remove blank plasma derived endogenous peaks, the extract was further purified by washing with tri-n-butyl phosphate and injected onto reversed-phase HPLC with ultraviolet detection at 333 nm. The precision and accuracy were less than 4.99% and within $\pm$7.89%, respectively. The limit of quantification was 10 ng/mL when 1 mL of plasma was used. The usefulness of this method was demonstrated by monitoring plasma concentrations after intravenous administration of YM872 to rats.

Keywords: YM872, Zonampanel monohydrate, AMPA Receptor antagonist, HPLC, UV detection, Plasma

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INTRODUCTION

The involvement of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), a glutamate receptor subtype, as well as N-methyl-D-aspartate (NMDA) glutamate receptor, in the development of ischemia induced neuronal damage has been demonstrated previously.[1] In a wide variety of animal models of cerebral ischemia, AMPA receptor antagonists have been shown to be neuroprotective.[2–4] As first generation AMPA receptor antagonists, quinoxalinedione derivatives such as 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline, have been shown to be neuroprotective against neuronal damage following focal cerebral ischemia.[5,6] However, the poor water solubility and resultant renal toxicity of these compounds may limit their experimental and clinical usefulness.[4] Zonampanel monohydrate, [2,3-Dioxo-7-(1*H*-imidazol-1-yl)-6-nitro-1,2,3,4-tetrahydro-1-quinoxalinyl]acetic acid monohydrate (YM872), is a novel AMPA receptor antagonist that is highly water soluble.[7,8] YM872 is expected to be an effective drug for the treatment of stroke or other conditions of acute neuronal degeneration that may involve glutamate neurotoxicity, without causing renal toxicity.

The radioactivity in rat plasma after intravenous administration of radiolabeled YM872 had a short half-life due to rapid elimination into the urine. For this reason, it was necessary to use a sensitive method to determine the plasma levels during the evaluation of the pharmacokinetic profile of YM872. This paper reports the development of a high performance liquid chromatography with ultraviolet detection (HPLC-UV) method for determining YM872, and the application of this method to rat plasma samples.

EXPERIMENTAL

Materials

YM872 and 3-[2,3-dioxo-7-(1*H*-imidazol-1-yl)-6-nitro-1,2,3,4-tetrahydro-1-quinoxalinyl]propionic acid monohydrochloride 0.7 hydrate, used as the internal standard, were synthesized at the Process Chemistry Laboratories and Chemistry Research Laboratories of Astellas Pharma Inc., respectively (Figure 1). Acetonitrile, methanol, and tri-n-butyl phosphate were purchased

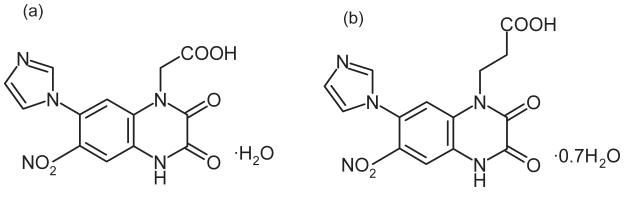

Figure 1. Chemical structure of YM872 (a) and the internal standard (b).

from Kanto Chemical (Tokyo, Japan). Tris[hydroxymethyl]aminomethane (Tris) and meglumine were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Purified water from a Milli-Q system (Millipore, Billerica, MA, U.S.A.) was used throughout the study, except for the preparation of the dosing solution. Water for injection was purchased from Otsuka Pharmaceutical (Tokyo, Japan). All other chemicals used were commercially available and of the highest purity.

Apparatus

The HPLC system consisted of a model LC-10AT pump (Shimadzu, Kyoto, Japan), a model SIL-10A auto-injector (Shimadzu), a model C-R7A integrator (Shimadzu), and a model SPD-10AV spectrophotometric detector. A mixture of 500 mM phosphoric acid, acetonitrile, and water (100:20:880, v/v/v) was used as the mobile phase. Separation was achieved on a TSK gel ODS-80Ts column (5 μm, 250 mm × 4.6 mm i.d., Tosoh, Tokyo, Japan) with a TSKguardgel ODS-80Ts (5 μm, 15 mm × 3.2 mm i.d., Tosoh) at a column temperature of 35°C. The HPLC system was operated at a mobile phase flow rate of 1.0 mL/min. The column eluate was monitored at 333 nm.

Preparation of Standard Solutions

A stock solution of YM872 at a concentration of 1 mg/mL was prepared by dissolving 10 mg of YM872 in 10 mL of 0.1 M hydrochloric acid. The stock solution was diluted with 0.01 M hydrochloric acid to prepare standard solutions for the calibration standards and quality control (QC) samples. A stock solution of the 1 mg/mL internal standard was prepared by dissolving 5 mg of the internal standard in 5 mL of 0.1 M hydrochloric acid. The stock solution was diluted with water (10-fold) and 0.01 M hydrochloric acid to prepare a 10 μg/mL solution.

Sample Preparation

A 0.1 mL aliquot of the internal standard solution (10 μg/mL) and 1 mL of 0.1 M Tris-HCl buffer (pH 7.0) were added to 1 mL of plasma in a 10-mL brown glass tube. The tube was mixed vigorously for 10 s. The mixture was applied to a solid phase extraction column (Sep-pak plus C_{18}, Waters, Midford, MA, U.S.A.), conditioned with 5 mL each of methanol, water, and 0.1 M Tris-HCl buffer (pH 7.0). After washing the column twice with 2.5 mL of 0.1 M Tris-HCl buffer (pH 7.0), the sample was eluted with 1 mL of 75% (v/v) methanol. The eluate was collected in a 10 mL brown glass tube. A 0.1-mL aliquot of 0.5 M hydrochloric acid was added to the eluate,

after which the mixture was washed twice with 6 mL of tri-n-butyl phosphate containing 5% (v/v) water. A 0.1 mL portion of the aqueous phase was injected into the HPLC.

Specificity

Plasma samples from six drug-free rats were extracted and assayed. The chromatograms were visually inspected for peaks from endogenous substances that might interfere with those of YM872 or the internal standard.

Calibration Curve

A 0.1 mL aliquot of each YM872 standard solution was added to 1 mL of blank rat plasma to prepare samples containing 10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 ng/mL of YM872. These standard samples were treated as described above. The peak height ratios of YM872 to the internal standard were plotted against nominal concentrations of YM872. The calibration curve was constructed using the least squares method, in which $1/(\text{concentration})^2$ was used as a weighting factor.

Sensitivity

The accuracy and precision of the assay method at the lower limit of quantitation were examined using six samples of rat plasma spiked with YM872 (10 ng/mL). Accuracy was expressed as the relative error, and precision as the relative S.D.

Accuracy and Precision

Intra- and inter-day accuracy and precision of the assay method were examined using rat plasma spiked with 30, 400, and 4000 ng/mL of YM872. Six samples were determined for each concentration. Accuracy was expressed as the relative error, and precision as the relative S.D.

Drug Administration

Male Sprague-Dawley rats were given free access to a standard pellet diet and tap water. YM872 was dissolved in water for injection containing meglumine, a solubilizer, at 1.3 times the molar ratio to YM872. The dosing solution was injected into the tail vein at a dose of 150 mg/kg ($n = 3$/time point). Blood

was collected from the inferior vena cava with a heparinized syringe under diethyl ether anesthesia. Plasma was separated by centrifuging the blood at 1,870 × g for 15 min, after which it was stored at −20°C until analysis.

RESULTS AND DISCUSSION

Method Development

YM872 possesses functional groups that contain amphoteric ions under physiological conditions. Accordingly, it exhibits high water solubility. This characteristic is favorable when avoidance of precipitation in the nephron, which hampers the clinical development of other AMPA receptor antagonists, is desired. In the search for an adequate assay method, it was found that liquid-liquid extraction, the most conventional sample pretreatment method, could not be used because of YM872's highly hydrophilic properties. In fact, several organic solvents such as ethyl acetate, n-hexane, tert-butyl methyl ether, and tri-n-butyl phosphate did not exhibit satisfactory extraction efficiency. In addition, no fluorescence detection method could detect YM872, despite the fact that this compound possesses a quinoxaline structure. Given these limitations, solid-phase extraction and ultraviolet monitoring were selected as the sample pretreatment and HPLC detection methods, respectively, for the bioanalysis of YM872. In the initial attempt to establish a bioanalytical method of YM872, the plasma sample eluate obtained via solid-phase extraction was evaporated to dryness for enrichment purposes. Unfortunately, during the 60 min elution period, endogenous substances were also eluted. Consequently, in a subsequent assay, the eluate was washed with organic solvent instead of evaporating it. Ethyl acetate, n-hexane, and tri-n-butyl phosphate were evaluated, with tri-n-butyl phosphate yielding the best results. Ethyl acetate was miscible with the eluate, which resulted in no phase separation, and no purification was achieved with n-hexane. The endogenous substances were effectively removed by tri-n-butyl phosphate. In addition, the methanol in the eluate was removed because of its miscibility in tri-n-butyl phosphate, which enabled a large volume of sample to be injected without causing peak broadening.

Specificity

Under the chromatographic conditions used, YM872 and the internal standard were eluted at 7.7 and 12.9 min, respectively (Figure 2). There were no interfering peaks of endogenous substances at the anticipated retention times of YM872 or the internal standard, which demonstrated the sufficient selectivity of the method.

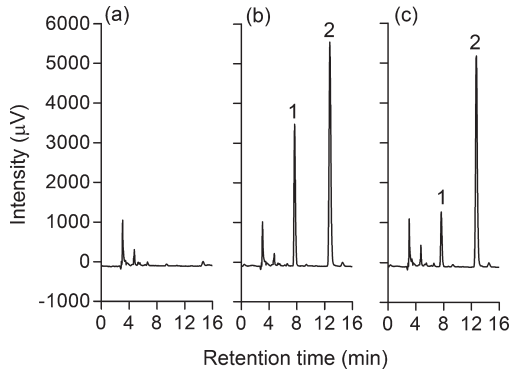

Figure 2. Typical chromatograms of rat plasma. (a) blank plasma; (b) blank plasma spiked with YM872 (500 ng/mL) and the internal standard (1000 ng/mL); (c) plasma sample obtained 2 h after intravenous administration of 150 mg/kg of YM872 (estimated concentration was 200.71 ng/mL). Peaks: 1 = YM872, 2 = internal standard.

Calibration Curve

Three calibration curves prepared on different days were compared. The calibration curves for YM872 in rat plasma were linear, using $1/\text{concentration}^2$ linear regression in the concentration range of 10 to 5000 ng/mL, with correlation coefficients greater than or equal to 0.9990. The overall precision for the three calibration curves obtained for YM872 in the calibration standards ranged between 0.92% and 2.48% relative S.D. (n = 3) (Table 1). For the calibration standards, all of the overall mean values (n = 3) were within $\pm 2.77\%$ of those expected (Table 1). For each calibration curve, the accuracy was within $\pm 5.67\%$.

Table 1. Mean back-calculated concentrations from three YM872 calibration curves

Concentration prepared (ng/mL)	Mean (n = 3)	S.D.	Relative S.D. (%)	Relative error (%)
10	9.82	0.12	1.24	−1.80
20	20.55	0.51	2.48	2.77
50	50.74	0.67	1.31	1.48
100	100.93	0.93	0.92	0.93
200	202.47	1.94	0.96	1.23
500	491.29	6.85	1.39	−1.74
1000	988.35	22.47	2.27	−1.17
2000	1980.19	21.23	1.07	−0.99
5000	4961.73	94.32	1.90	−0.77

Sensitivity

At the lower limit of quantification for YM872 (10 ng/mL), the relative S.D. (n = 6) of the measured concentrations was 4.51%, and the accuracy was 5.36%.

Accuracy and Precision

Intra-day precision for YM872 in rat plasma ranged from 0.70% to 3.03% relative S.D. (n = 6) at the three QC sample concentrations (30, 400, and 4,000 ng/mL) (Table 2). Overall precision ranged from 2.53% to 4.99% relative S.D. (n = 18) for the QC samples (inter-day precision in Table 2). The daily mean values (n = 6) of the QC samples were all within ±7.89% of their expected values (intra-day accuracy in Table 2). All of the overall mean values (n = 18) were within ±2.34% of their expected values (inter-day accuracy in Table 2).

Application to Pharmacokinetic Studies

The usefulness of the method was demonstrated by monitoring plasma concentrations of the unchanged drug after the intravenous administration of YM872 to rats at 150 mg/kg (Figure 3). The drug was eliminated

Table 2. Accuracy and precision of YM872 QC samples

Concentration prepared (ng/mL)	Intra-day (n = 6)			Inter-day (n = 18)
	1	2	3	
30				
Mean (ng/mL)	30.74	32.37	29.00	30.70
S.D. (ng/mL)	0.41	0.86	0.53	1.53
Relative S.D. (%)	1.34	2.64	1.81	4.99
Relative error (%)	2.46	7.89	−3.33	2.34
400				
Mean (ng/mL)	403.11	407.71	390.85	400.56
S.D. (ng/mL)	2.84	7.72	10.02	10.15
Relative S.D. (%)	0.70	1.89	2.56	2.53
Relative error (%)	0.78	1.93	−2.29	0.14
4000				
Mean (ng/mL)	4045.13	4199.88	3816.45	4020.49
S.D. (ng/mL)	51.38	127.41	95.42	185.73
Relative S.D. (%)	1.27	3.03	2.50	4.62
Relative error (%)	1.13	5.00	−4.59	0.51

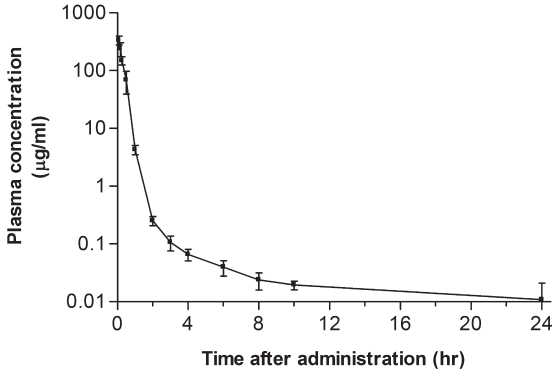

Figure 3. Plasma concentration of the unchanged drug after intravenous administration of YM872 at a dose of 150 mg/kg to rats. Values are expressed as the mean ± S.D. of three rats.

biphasically, and could be detected by this method, even at the last sampling point.

CONCLUSION

A method was developed for the determination of YM872, a novel neuroprotective drug, in rat plasma using HPLC. The method consists of sample preparation by solid-phase extraction, clean-up with organic solvent, chromatographic separation, and UV detection. The method is simple and sensitive enough for the evaluation of plasma concentration profiles after intravenous administration of YM872 to rats.

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