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Determination of a novel low-voltage-activated calcium channel blocker (HYP-10) in rat plasma by liquid chromatography–mass spectrometry

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ABSTRACT

A novel T-type calcium channel blocker, 4-amino-1-{4-[(4-chloro-phenyl)-phenyl-methyl]-piperazin-1-yl}-butan-1-one (HYP-10) has been synthesized, and the compound has shown promise as both a nociceptive and inflammatory pain reliever as well as an analgesic in a rat neuropathic pain model. A quantification method was developed for the determination of HYP-10 in rat plasma. After simple protein precipitation with methanol, HYP-10 and the internal standard, methaqualone were chromatographed on a reversed-phase column and detected by liquid chromatography/tandem mass spectrometry with electrospray ionization. The accuracy and precision of the assay were in accordance with FDA regulations for validation of bioanalytical methods. This method was applied to measure the plasma HYP-10 concentration after a single intravenous administration of the compound in rats.

1. Introduction

Low-voltage-activated (LVA or T-type) calcium channels are known to have a strong association with absence seizure [1], sleep disorders [2] and pain [3]. The three genes corresponding to the α_1 subunit of the T-type calcium channel are $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$ [4]. Among the three isoforms of the T-type channel, $Ca_v3.1$ is expressed in peripheral and central neurons of the pain pathway and plays a pivotal role in the mediation of physiological and pathological pain [3].

Recently, our group synthesized a novel T-type calcium channel blocker, 4-amino-1-{4-[(4-chloro-phenyl)-phenyl-methyl]-piperazin-1-yl}-butan-1-one (HYP-10, Fig. 1). It possesses a diphenylpiperazine moiety with a flexible alkyl chain, attached with hydrophobic groups, that acts as a linker, mimicking flunarizine and pimozide. In a rat neuropathic pain model, HYP-10 showed promise as a nociceptive and inflammatory pain inhibitor as well as an analgesic by blocking T-type calcium channels [5].

Together with the evaluation of pharmacodynamic properties, a series of pharmacokinetic studies has to be performed from the very beginning of any drug development process. Therefore, in this study, we developed an analytical method for the determination of HYP-10 in rat plasma using liquid chromatography coupled to tandem mass spectrometry. As a part of a preclinical drug development process, pharmacokinetic profiles of HYP-10 have been examined in rodents, resulting in approximately 50% of absolute bioavailability following intravenous injection and oral administration of the compound. In addition, single and repeat-dose toxicology studies in animals are in progress to assess safety of the compound. The present method was fully validated and applied to pharmacokinetic study following intravenous injection of HYP-10 into rat.

2. Experimental

2.1. Reagents and materials

HYP-10 was synthesized at a medicinal chemistry laboratory at Ewha Womans University, Korea. The internal standard (IS), methaqualone and formic acid were purchased from Sigma (Seoul, Korea), and methanol was obtained from J.T. Baker (Seoul, Korea). All other chemicals and solvents were of the highest analytical grade available.

2.2. Preparation of standards and quality controls

HYP-10 was dissolved in methanol and distilled water (50:50) to obtain a concentration of 1 mg/ml. The stock solution was serially diluted further, and 50 μl of each solution was added to 950 μl of drug-free plasma to obtain the final concentrations of 10, 20,

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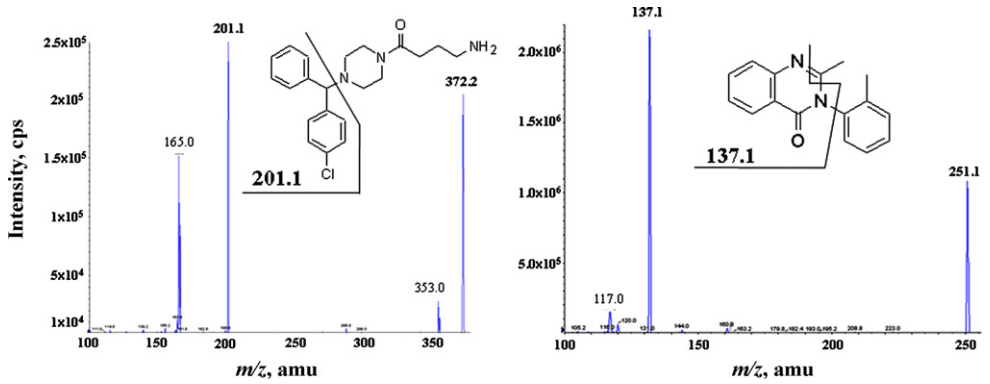

Fig. 1. Structures and product ion spectra of a novel low-voltage-activated calcium channel blocker, HYP-10 (left) and methaqualone (right).

50, 100, 500 and 5000 ng/ml. 100 μl of aliquot was taken from the spiked plasma for calibrations. Using linear regression, five calibration graphs were derived from the ratio between the area under the peak of each compound and the IS.

Quality control samples were prepared in 950 μl of blank rat plasma by adding 50 μl of serially diluted solutions of HYP-10 to obtain the lower limit of quantification (10 ng/ml) as well as the low (30 ng/ml), intermediate (300 ng/ml), and high (4000 ng/ml) concentrations of the control samples. An aliquot (0.1 ml) was taken from the spiked plasma. These samples were used to evaluate the intra- and inter-day precision and accuracy of the assay.

2.3. Analytical system

Plasma concentrations of HYP-10 were quantified using the API 4000 LC–MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization interface that was used in the positive ion mode, $[M+H]^+$.

The compounds were separated on a reverse-phase column (Atrantis T3, 50 mm × 2.1 mm internal diameter, 3 μm particle size; Waters, USA) with a Security Guard cartridge (4 mm × 2.0 mm internal diameter; Phenomenex, USA). The mobile phase was 0.1% formic acid: methanol (3:7, v/v). The column was maintained at 35 °C, and the mobile phase was eluted at 0.3 ml/min using HP 1100 series pump (Agilent, Wilmington, DE, USA). The Turbolon Spray (Applied Biosystems) interface was operated in the positive ion mode at 5500 V and 450 °C. HYP-10 and the IS, methaqualone produced mainly protonated molecules at *m/z* 372.2 and 251.1, respectively. The product ions were scanned in Q3 after collision with nitrogen in Q2 at *m/z* 201.1 for HYP-10 and at *m/z* 131.7 for IS. Quantitative analysis was performed by multiple reaction monitoring of the protonated precursor ions and the related product ions using the ratio of the area under the peak for each solution and a weighting factor of $1/y^2$. The analytical data were processed with Analyst® software (version 1.4.1, Applied Biosystems).

2.4. Sample preparation

300 μl of the IS (10 ng/ml in methanol) was added to 100 μl of rat plasma, vigorously mixed for 30 s and spun in a centrifuge at 13,200 rpm for 10 min to precipitate the protein. Finally, 3 μl of the supernatant was injected onto the column.

2.5. Validation procedure

The validation parameters were selectivity, precision, and accuracy. Blank plasma samples obtained from five rats were screened to determine specificity. The intra- and inter-day assay precision and accuracy were estimated using a calibration curve to predict the concentration of the quality controls. Acceptable criteria were within 15% of precision and accuracy, except the lower limit of quantification was within 20%.

The recovery was determined by comparing the mean peak areas of quality controls spiked before protein precipitation to those spiked after the pretreatment. The matrix effect was assessed by a percentile of the mean peak areas of quality controls spiked after the pretreatment to those of stock solutions.

2.6. Stability

Stability was examined in stock solution and plasma samples under different conditions. The stock solution was checked for short-term stability after 4 h of storage at room temperature and for long-term stability after 2 weeks at 4 °C.

For the stability study in plasma, control samples were made into 10, 100 and 4000 ng/ml concentrations. Short-term stability was assessed after 4 h of storage at room temperature; long-term stability was assessed after 2 weeks of storage in a freezer at −20 °C. The stability of HYP-10 in plasma samples was tested after three freeze–thaw cycles (−70 °C to room temperature). The stability of HYP-10 in extracts was also examined after 24 h of storage at 4 °C. The requirement for a stable analyte was that the difference between the mean concentrations of tested samples after being placed under various conditions had to be in the ±15% range.

2.7. Pharmacokinetic application

Five male rats were involved in a pharmacokinetic study approved by the Institutional Animal Ethics Commitee of Ewha Womans University, Korea. After an overnight fast, all rats were given a single intravenous dose of 5 mg/kg HYP-10. About 0.2 ml of heparinized blood was collected from the common carotid artery for a designated time period after drug administration. Blood samples were centrifuged immediately, and the plasma was collected and stored at −20 °C until analyzed.

3. Results and discussion

3.1. Quantification of HYP-10 and validation of method

Fig. 1 shows the precursor and corresponding product ions for HYP-10 and the IS. The instrument was operated in the positive ion mode with N_2 collision gas in Q2 of the MS/MS system. HYP-10 and the IS predominantly produced protonated molecules at *m/z* 372.2 and 251.1, respectively. After collision with N_2 in Q2, the corresponding product ions were scanned at *m/z* 201.1 and 131.7, respectively, in Q3 [6]. These were the most sensitive product ions for quantification.

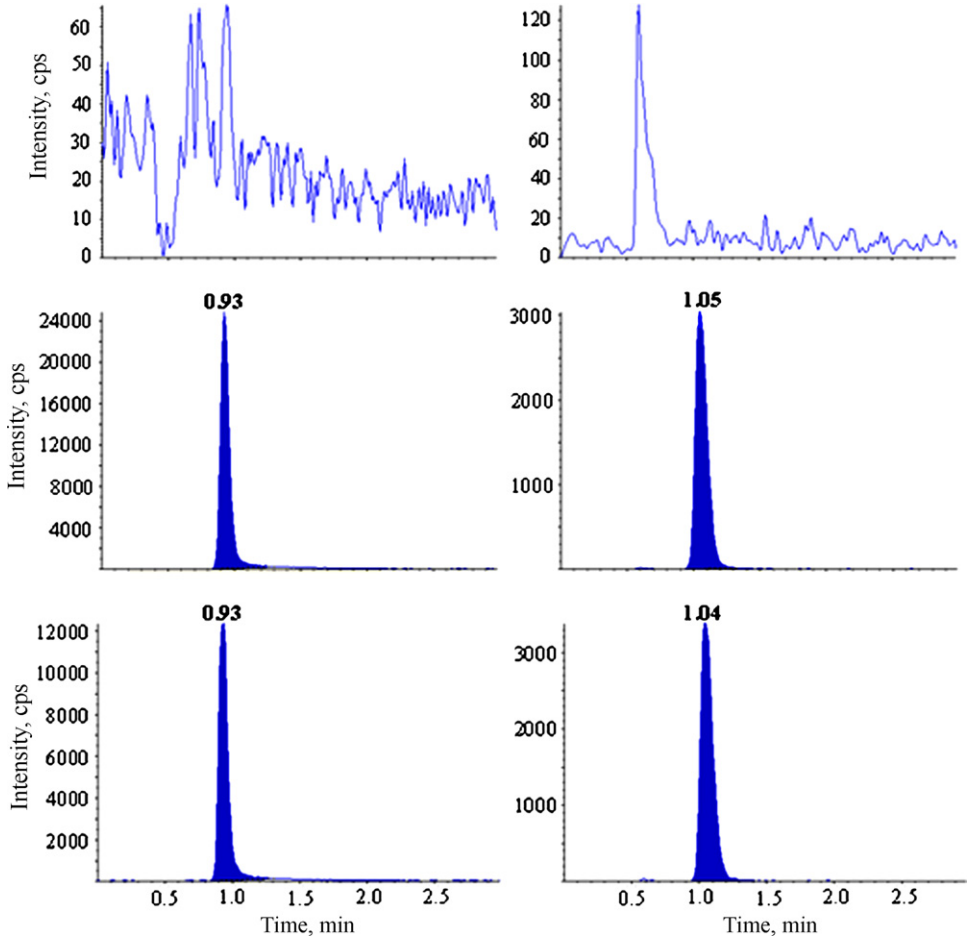

Fig. 2. Chromatograms of HYP-10 and methaqualone. Top, double-blank plasma; middle, plasma spiked with 100 ng/ml HYP-10 and 10 ng/ml methaqualone (IS); bottom, a plasma sample equivalent to 53.1 μg/ml for HYP-10 obtained from a rat at 2 h after an intravenous administration of 5 mg/kg HYP-10.

There were no interfering peaks at the elution times for either the HYP-10 or the IS. Fig. 2 presents typical chromatograms for the blank plasma (top), plasma spiked with 100 ng/ml of HYP-10 plus 10 ng/ml IS (middle), and a rat plasma sample (bottom). The calibration curves provided a reliable response for HYP-10 (10–5000 ng/ml, $r^2 > 0.999$). The ratio of the peak area of HYP-10 relative to that of the IS correlated with the corresponding plasma concentration, and good linearity was observed. The detection limit for HYP-10 was 1 ng/ml at a signal-to-noise (S/N) ratio of 3. The estimates of the intra- and inter-day precision and accuracy of the assay are presented in Table 1. The relative standard deviations of the intra- and inter-day assay precision were less than 4.1% and 7.0%, respectively. The intra- and inter-day assay accuracy was 100.7–103.5% and 97.5–105.6%, respectively. The mean recovery of HYP-10 exceeded 96% for the assays, and the mean matrix effect was about 31% (Table 2).

Table 1
Precision and accuracy of the intra- and inter day assay ($n = 5$).

Concentration (ng/ml)	Intra-day	Inter-day
10	103.1 ± 2.5[a] (2.4)[b]	105.6 ± 6.1 (5.8)
30	102.7 ± 1.9 (1.9)	98.7 ± 3.7 (3.7)
300	103.5 ± 3.7 (3.6)	105.5 ± 2.5 (2.4)
4000	100.7 ± 4.1 (4.1)	97.5 ± 6.8 (7.0)

[a] Accuracy (mean % ± S.D.).
[b] R.S.D., relative standard deviation (%).

Table 2
Matrix effect and recovery for HYP-10 in rat plasma.

Concentration (ng/ml)	Matrix effect (%)	Recovery (%)
10	34.5	98.7
30	29.8	98.6
300	30.2	96.7
4000	29.2	96.4

3.2. Stability

The stabilities of drug and IS in a biological matrix are affected by the chemical properties of the compound, storage conditions and container systems. The stability tests are designed to reflect the condition during sample handling and analysis. HYP-10 stock solution was stable at room temperature for 4 h as well as at 4 °C for 2 weeks. HYP-10 in plasma was stable up to 4 h at room temperature; it also remained intact at −20 °C for up to 2 weeks. No degradation was observed after three cycles of freezing and thawing. The stability of HYP-10 in extracts was confirmed after 24 h of storage at 4 °C (Table 3). On the basis of the results of stability tests, HYP-10 was found to be stable during sample collection, handling and analysis.

3.3. Application of the method

The validated method was used to evaluate the pharmacokinetics of HYP-10 in rats. Fig. 3 shows the mean plasma

Table 3
Stability of HYP-10 after storage under indicated condition (mean % ± S.D., $n = 3$).

Storage condition	10 ng/ml	100 ng/ml	4000 ng/ml
Standard solutions			
Room temperature (4 h)	101.7 ± 1.1	100.6 ± 2.9	97.2 ± 3.1
Refrigerator (2 weeks)	96.1 ± 1.2	96.8 ± 2.1	102.4 ± 4.6
Plasma samples			
Room temperature (4 h)	105.5 ± 5.5	97.2 ± 4.4	96.7 ± 2.2
Refrigerator in extracts (24 h)	106.1 ± 4.4	106.3 ± 3.9	107.7 ± 6.1
3 cycles of freezing-thawing	107.0 ± 5.8	107.6 ± 4.7	98.1 ± 3.6
−20 °C (2 weeks)	105.4 ± 6.5	95.8 ± 5.9	101.9 ± 7.5

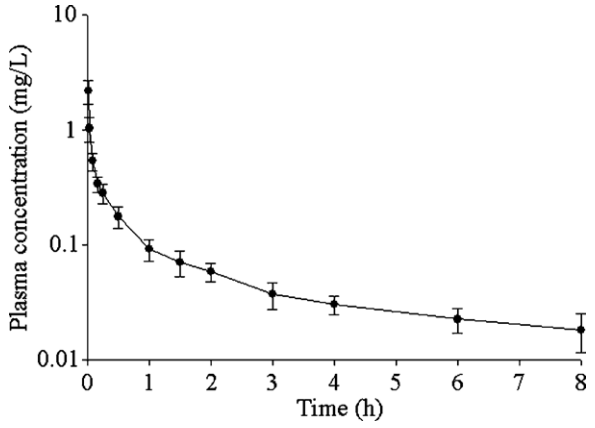

Fig. 3. Time course of plasma HYP-10 concentrations after a single intravenous administration of 5 mg/kg HYP-10 in rats ($n = 5$). Each point represents mean ± S.D.

concentrations after a single intravenous dose of 5 mg/kg HYP-10 in rats.

In conclusion, a specific and sensitive method for the determination of a novel low-voltage-activated calcium channel blocker, HYP-10, in rat plasma was developed using LC–MS/MS. This method is suitable for pharmacokinetic studies of the compound in vivo.

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