SHIMADZU

MP-102

Quantitation of Sazetidine-A for Tinnitus Management in Rat Blood after Oral, Intraperitoneal, and Subcutaneous Injections

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1. Overview

Tinnitus is a condition commonly described as a phantom ringing in the ears. This condition can often be debilitating as the symptoms can lead to secondary symptoms including anxiety and depression. Currently there is no cure for tinnitus and treatments are limited to reducing the perception of the symptoms. There are a limited number of drugs that can be used to reduce the symptoms, including antidepressants and nicotinic acetylcholine receptors. Sazetidine-A (SAZ-A) is being investigated as a possible new drug due to its partial agonist resulting in decreased anxiety and depression as well as pain relief. In order to pursue SAZ-A use in rats, revision of the current methods of drug administration, which can induce excess stress on the animal resulting in compilations and higher mortality rates, was necessary. A new wafer (BIO-Serv) was tested against the traditional methods of dosing.

2. Introduction

SAZ-A acts as a partial agonist and is known to desensitize neuronal nicotinic acetylcholine receptors (nAChR). When the nAChR receptor is desensitized, the receptor is not activated by acetylcholine. This can attribute to pain relief, and reduced levels of anxiety and depression. The administrations of SAZ-A to the animals in recent behavioral studies were via intraperitoneal, subcutaneous or oral gavage. These administration routes may interfere with the behavioral testing by stressing the animals. A non-stress oral administration of SAZ-A was developed by feeding rats with a wafer (BIO-Serv) dosed with SAZ-A. Here, we determine the optimal starting dose by quantifying the level of Sazetidine-A in the blood after dosing by different routes of administration.

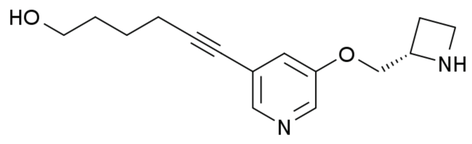

Figure 1. Structure Sazetidine-A

3. Methods

3-1 Sample Preparation

Fifteen Long Evan (LE) rats (9-10 mos. age) were dosed with 1 mg/kg or 3 mg/kg SAZ-A via either wafer, IP or SubCu. Rats were anesthetized with isoflurane; tail or heart blood was drawn at 15 minutes and 1 hour after SAZ-A administration. Six samples were collected from the heart and 18 samples were collected from the tails of the LE rats. Plasma was collected after 1,500 × g 15 minutes centrifugation and stored in -80° C until use. Samples, comprised of 100 µL plasma and 10 µL internal standard (caffeine), were filtered through 5 kDa filters. Liquid-liquid extraction was performed using chloroform and ethyl acetate. The supernatant was transferred to a new vial and dried under nitrogen followed by reconstitution in a 100 mM ammonium acetate solution.

High Speed Mass Spectrometer

Ultra Fast Polarity Switching
- 5msec

Ultra Fast MRM
- Max. 555 transition /sec

Figure 2. LCMS-8060NX triple quadrupole mass spectrometer

3-2 Instrumental Analysis

An LC-30 Nexera HPLC system was coupled to an LCMS-8060NX triple quadruple mass spectrometer with electrospray ionization (ESI) shown in Figure 2. All compounds were analysed in positive polarity using MRM mode. The mobile phase composition and LC and MS interface parameters are shown below. MRM transitions are shown in Table 1.

A calibration curve of SAZ-A was created in a 100 mM ammonium acetate solution from 1 µg/kg -1000 µg/kg , with an internal standard of caffeine at 100 µg/kg . The calibration curve and samples were analysed in triplicate with a 1 µL injection volume with a 13-minute method.

UHPLC conditions (LC-30 Nexera system)

Column: Phenomenex Synergi Polar-RP 150 mm × 4.6 mm, 4 µm, 80Å
Mobile phase A: 10 mM ammonium acetate/ 0.1% acetic acid in water
B: 10 mM ammonium acetate/ 0.1% acetic acid in methanol
Flow rate: 0.7 mL/min
Time program: B conc.20%(0 min) -90%(0-3min) - 95%(3-6.5min)-100%(6.5-7min). 100%(7-10min)-20%(10.1-13min).
Injection vol.: 1 µL
Column temperature: 45° C

MS conditions (LCMS-8060NX)

Ionization: ESI, Positive MRM mode

Interface Parameters:
Nebulizing Gas: 3 L/min
Heating Gas: 15 L/min
Drying Gas: 3 L/min
Interface Temp: 350° C
Desolvation Temp: 200° C
Heat Block Temp: 200° C
Interface Voltage: 1 kV
Focus Voltage: 2 kV

Table 1. MRM transition of SAZ-A and caffeine. Quantitative transitions are bolded.

Compound	MRM transition	Collision Energy
SAZ-A	**261.20>70.20**	**-30.0**
	261.20>192.10	-12.0
	261.20>232.20	-11.0
	261.20>133.10	-23.0
Caffeine	**195.10>138.10**	**-20.0**
	195.10>110.15	-23.0
	195.10>42.15	-35.0

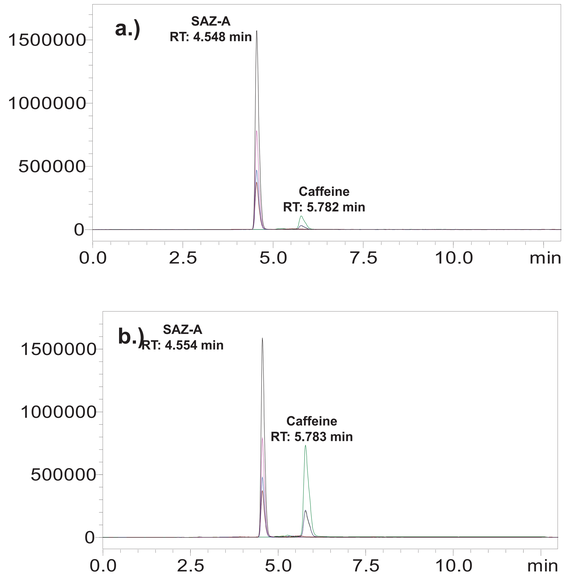

Figure 3. Chromatogram of SAZ-A and caffeine in, a.) blood plasma matrix quantified at 0.246 µg/kg , b.) ammonium acetate solution at 0.250 µg/kg .

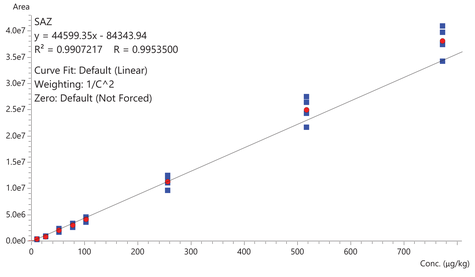

Figure 5. Calibration curve of SAZ-A in ammonium acetate solution used to calculate matrix samples. The calibration curve ranges from 1 µg/kg - 1000 µg/kg , with an R^2 of 0.99.

4. Results

The development of an LCMS method for the selective quantitation of SAZ-A from rat serum was completed. Because of the low levels of drug concentration observed in serum, the sensitivity of the method was paramount to the success of the biological sample analysis. Figure 3 shows chromatograms from a typical sample (**a**) and a standard (**b**). These are shown to demonstrate the consistency between standard and sample for retention times as well as the use of MRM in the analysis. Figure 4 exhibits the chromatogram for the standard illustrating the limit of quantitation. Figure 5 displays the calibration curve covering three orders of magnitude with an R^2 value of >0.99.

The real world sample data are listed in Tables 2 and 3 showing individual sample data and summary data, respectively. The summary data indicates that dosing with a wafer results in the same order of magnitude serum level using 1 mg/kg by wafer and SC or by increasing the wafer concentration to 3 mg/kg and collecting after 15 min while using a 1 mg/kg SC dosing. This is an important result as the tinnitus study moves forward using the wafer method to minimize rat stress during dosing in order to study the effect of SAZ-A.

Table 2. Sample data for individual Long Evan Rats dosed with SAZ-A via wafer and SubCu injection.

Trial 3- 1 mg/kg SAZ-A			
Sample	**Time (min)**	**Route**	**Concentration (µg/kg)**
DOD-188	60	Wafer	14.54
DOD-189	15	SC	240.38
DOD-190	60	Wafer	15.48
DOD-191	60	Wafer	14.11
DOD-193	60	SC	75.40
DOD-194	60	SC	28.54
DOD-195	15	SC	205.05
DOD-196	60	SC	13.64
DOD-197	15	SC	234.97

Table 3. Summary data for LE rats dosed with 1 mg/kg and 3 mg/kg via the three routes tested.

1mg/kg 15 min	IP (µg/kg)	Wafer (µg/kg)	SC (µg/kg)
Trial 1	382.8	17.15	
Trial 2			226.8
1mg/kg 1 hour			
Trial 1	182.7	21.84	
Trial 2		14.71	39.20
3mg/kg 15 min			
Trial 1	1125	285.9	
***Time: Time to collect blood after drug administration.**			