

Determination of 5-HT_{2C} agonist KKHQ80114 and KKHQ80109 in Rat Plasma and Urine by Gas-Chromatography/Mass Spectrometry

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ABSTRACT – 5-HT_{2C} receptors have been considered as therapeutic targets for the treatment of various central nervous system disorders such as depression, anxiety, epilepsy, schizophrenia and sleep disorders. We chemically synthesized KKHQ80114 (K14) and KKHQ80109 (K09), selective 5-HT_{2C} agonists, with the purpose of developing therapeutic agents for the treatment of obesity. The objective of this work is to investigate analytical methods of these compounds in the plasma and urine of rats by gas chromatography/mass spectrometry. In this experiment, K14 was determined in plasma and urine by using K09 as internal standard. Calibration curves give a good linearity in plasma ($r^2=0.9993$) and urine ($r^2=0.9988$). Among hexane, ethyl acetate and diethyl ether, the highest peak was observed in diethyl ether. However, ethyl acetate was used since more interfering peaks were observed with diethyl ether. Inter-day precision and accuracy were determined in the ranges of 50-500 ng/mL for plasma and 10-500 ng/ml for urine. Quantitation limits were 50 ng/mL plasma and 25 ng/mL urine. These data may be applicable for further studies of these compounds including absorption and metabolism due to no pharmacokinetic or analytical data available.

Key words – KKHQ80114, KKHQ80109, Gas chromatography/mass selective detector, Plasma, Urine, Rats.

5-Hydroxytryptamine (5-HT) is well known to be widely distributed in the central and peripheral nervous systems in mammals and to play important roles in regulation and modulation of physiological and behavioral functions. There found at least 14 different 5-HT receptor subtypes that are classified into 7 sub-family from 5-HT₁ to 5-HT₇.^{1,2)}

Among these receptors, 5-HT_{2C} receptors have been considered as therapeutic targets for the treatment of various central nervous system disorders such as depression, anxiety, epilepsy, schizophrenia and sleep disorders.³⁻⁸⁾ 5-HT_{2C} receptor mRNA is found in brain regions of the rat that are involved in feeding behavior, including choroids plexus, the nucleus of the solitary tract, dorsal medial hypothalamic nucleus, paraventricular hypothalamic nucleus, amygdala and other brain regions related with the regulation of appetite.⁹⁻¹⁰⁾

5-HT_{2C} agonists have been reported to exert selective anti-obesity effects. However, the limit to the development of anti-obesity drugs up to the present does not possess good selectivity and specificity of drug candidates to the 5-HT_{2C} receptor because they also activate 5-HT_{2A} and 5-HT_{2B} receptors, resulting in various side effects associating to these receptors.

This is also due to the structural similarity of the 5-HT_{2C} receptor to 5-HT_{2A} and 5-HT_{2B} receptors since it has very high homology (49-50%) in entire domain and identity (71-80%) of amino acid sequences in the transmembrane domain.¹¹⁻¹²⁾

Therefore, anti-obesity drug candidates are greatly focused on developing more selective and specific 5-HT_{2C} agonists.

We chemically synthesized KKHQ80114 (K14; N-(benzyl)-N-(1-ethyl-piperidine)-3-trifluoromethylbenzenesulfonamide) and KKHQ80109 (K09; N-benzyl-N-(2-(2-ethylpiperidin-1-yl)ethyl)-4-propylbenzenesulfonamide hydrochloride), selective 5-HT_{2C} agonists (unpublished report), with the purpose of developing therapeutic agents for the treatment of obesity. Analytical methods of these compounds in the biological fluid are required for pharmacokinetic study. The sensitive methods of K114 and K09, however, are not available. The objective of this work is to develop sensitive analytical methods in the plasma and urine of rats by gas chromatography/mass spectrometry for pharmacokinetic studies and metabolism.

Materials and Methods

Chemicals

KKHQ80114 (K14), KKHQ80109 (K09, internal standard) and other analogues were chemically synthesized by the KIST chemoinformatic group. Ethyl acetate, diethyl ether, hexane and

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methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). The other agents used for K14 and K09 analysis were of analytical grade.

Animal Treatment for Blood and Urine Sampling

Male Sprague-Dawley rats (230±15 g) were purchased from Orient Bio (Chungbuk, Korea). The rats were acclimatized in the KIST animal facility for at least 1 week under the control of constant temperature and humidity. A 12-hr light/12-hr dark cycle was maintained beginning at 6:00 hr. Milipore-filtered tap water and feed (Samyang Co., Seoul, Korea) were provided *ad libitum*. The Internal Animal Care and Use Committee approved animal handling procedure for the experiment.

One day before the pharmacokinetic study, the rats were received surgery for catheterization of the carotid artery after anesthesia. Polyethylene tube (PE-50) was inserted to the carotid artery and the catheter passed subcutaneously to the dorsal nape of the neck, maintaining the catheter with heparin and holding the rat into metabolic cages through the experimental period.

Gas-chromatography/ Mass Selective Detector

The plasma concentrations of K14 in rat plasma were determined by a gas chromatography/mass selective detector (GC/MSD; HP 6890 Series/5972; Hewlett-Packard, CA, USA). The samples were injected to the instrument by an autoliquid sampler (Agilent 7983 Series), being supported with the GC/MSD ChemStation (Kayak PC/G1701DA, Hewlett Packard, USA). Mass selective detector of electron impact mode and selected ion monitoring mode were used, and ionized energy of the mode was 70 eV. The K14 was separated by using the column Ultra-2 (17 m length × 0.2 mm inner diameter × 0.33 μm film thickness; Agilent Technologies, USA). Initial temperature of the oven was set to 180°C, at which the temperature was increased by a rate of 30°C per min to 260°C without holding time, and increased at a rate of 10°C per min to 300°C of the final temperature where stayed for 12 min. Temperatures of inlet, transfer line and detector were all set to 300°C. The flow rate of helium as carrier gas was 0.8 ml/min. The characteristic ions selected were *m/z* 91, 126, 245 and 441 for K14, and *m/z* 91, 126, 245 and 399 for internal standard K09. The ion of *m/z* 126 was used for the quantitation of K14 and K09.

Preparation of the Calibration Curve of K14

To 0.1 mL of the K14 and K09-free plasma or 1 mL blank urine, K14 was added to plasma to make final concentrations of 0, 50, 75, 100, 250 and 500 ng/mL each and K09 (10 μg/ml, 20 μL) as internal standard to glass-centrifuged tubes with

stopper. For the urine samples, K14 was added at the final concentrations of 10, 25, 50, 100, 250 and 500 ng/mL. The tubes were vortex-mixed, and 0.1 mL of 0.5 N potassium carbonate and 0.5 mL of distilled water were added and agitated. After addition of 5 mL ethyl acetate, the tubes were shaken for 20 min on a shaker (100-150 rpm; 7400 Tubingen, Edmund Buchler, Germany) and centrifuged at 2500 rpm (900 g) for 10 min (Varifuge 3.0, Heraeus, Germany). The organic layer was transferred to a new tube after freezing the tube in a freezer (-30°C, Ecoline RE112, Lauda, Germany). Ethyl acetate was evaporated by a nitrogen evaporator (TurvoVap, Zymark, Hopkinton, MA, USA) and the tube was placed in a desiccator with potassium pentoxide/ potassium hydroxide for at least 3 hr. The residue was dissolved in 50 mL of methanol, and 2 mL of the solution was injected to GC/MSD by an auto liquid sampler. The method was validated by measuring inter-day precision and accuracy in the plasma and urine.

Clean-up of Plasma and Urine Samples

The plasma and urine samples obtained from rats were thawed at room temperature. To the tube, 0.1 mL of the plasma and 1 mL of the urine samples were added to glass-centrifuge tubes with stopper with the internal standard K09 (10 μg/mL, 20 μL) spiked. The tubes were vortex-mixed, and 0.1 mL of 0.5 N potassium carbonate and 0.5 mL of distilled water were added and agitated. After addition of 5 mL ethyl acetate, the tubes were shaken for 20 min on a shaker and centrifuged at 2500 rpm (900 g) for 10 min. The organic layer was transferred to a new tube after freezing the tube in a freezer (-30°C, Ecoline RE112, Lauda, Germany). Ethyl acetate was evaporated by a nitrogen evaporator and the tube was placed in a desiccator with potassium pentoxide/ potassium hydroxide for at least 3 hr. The residue was dissolved in 50 ml of methanol, and 2 μL of the solution was injected to GC/MSD by an auto liquid sampler. The plasma and urine concentrations of K14 in rats were determined, based on the calibration curve from peak area ratios of K14 to the internal standard.

Results and Discussion

The GC/MSD scan spectra of K14 and K09 (with their chemical structures also in Figure 1) were showed in Figure 2. The base peak ions were *m/z* 126 that is the identical ion for both K14 and K09 with another common ion of *m/z* 245. The characteristic ions of K14 and K09 were *m/z* 441 and *m/z* 399 as results from the fragmentation [$M^+ - C_2H_5$], respectively. The common base peak ion of *m/z* 126 was selected for quantitation of K14 and internal standard K09. Retention times of

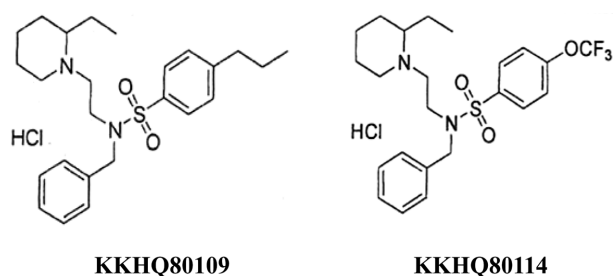


Figure 1—Chemical structures of KKHQ80109 (K09) and KKHQ80114 (K14).

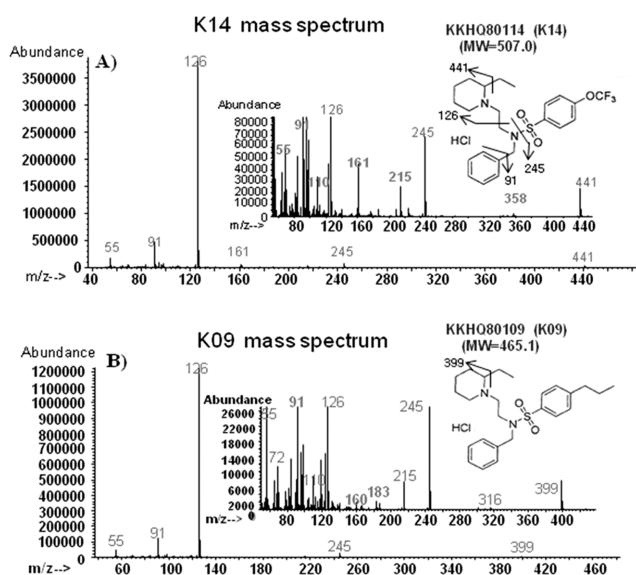


Figure 2—GC/MSD scan spectra of K09 and K14. Magnified scale of the mass spectra was showed in the inset and mass fragmentation for major characteristic ions was indicated within the chemical structures with *m/z* 126 of base peaks. Characteristic ions of *m/z* 441 and *m/z* 399 were derived by cleavage of [C₂H₅] from M⁺ of free forms of K14 and K09.

K14 and K09 were observed to be 6.7-7.0 and 10.2-10.8 min, respectively (Figure 3). The chemical structures of K14 and K09 are very similar except for one moiety on benzylsulfonamide skeleton, respectively, as trifluoromethyl and propyl groups. The minor difference in the chemical structures of these compounds resulted in the production of mass fragmentation of the identical ions that enable these compounds to unambiguously interpret the mass spectrum of two compounds. K09 is also considered a good internal standard for K14 determination due to very close similarity of the structure, and also reversely.

The pharmacokinetic study in rats requires the sensitive analytical methods because of limitation of small amount of the blood from rats. The method was validated by measuring precision and accuracy in the plasma and urine of rats. As indi-

Table I—Inter-day precision and accuracy for the determination of K14 in rat plasma

K14 Concentrations (ng/ml plasma)	C.V. (%) ^a	Accuracy ^a (%)
50.0	9.10	102.30
75.0	2.70	102.96
100.0	9.03	106.16
250.0	3.06	96.54
500.0	4.31	100.49

^aEach value was showed as mean of 5 replicates.

Table II—Inter-day precision and accuracy for the determination of K14 in rat urine

K14 Concentrations (ng/ml urine)	C.V. (%) ^a	Accuracy ^a (%)
25.0	9.94	111.98
50.0	9.85	95.12
100.0	3.07	89.10
250.0	1.62	98.42
500.0	11.89	101.21

^aEach value was showed as mean of 5 replicates.

cated in Table I, precision for the determination of K14 in the plasma was less than 9.10% and accuracy had 2.30% of bias at 5 ng/100 μL (50 ng/mL) with less than 6.16% of bias. Precision in urine samples were less than 11.89%, and accuracy was 41.04 % at the lowest concentration of 10 ng/mL with less than 11.89% at the rest of concentrations (Table II). Quantitation limits of K14 in plasma and urine were 5 ng/100 μL and 25 ng/mL, respectively. The calibration curves of K14 showed good linearity for plasma ($r^2=0.9993$; $y=0.0071x + 0.0177$) and urine ($r^2=0.9988$; $y=0.0061x - 0.0051$), as showed in Figure 4.

To investigate an optimal solvent for K14 and K09 determination, hexane, ethyl acetate, and diethyl ether were compared for the extraction of K14 and K09 (Figure 5). The highest extraction of K14 and K09 were observed with diethyl ether, and the next was with ethyl acetate and hexane. Even if the highest extraction was occurred in diethyl ether, in our experiment ethyl acetate was selected as extraction solvent since more peaks were found in the chromatograms with diethyl ether, compared to those of ethyl acetate (data not shown).

Binding affinity of K14 and K09 was observed to be 1232 and 909 nM for 5-HT_{2a} and 91 and 49 for 5-HT_{2c} receptors,

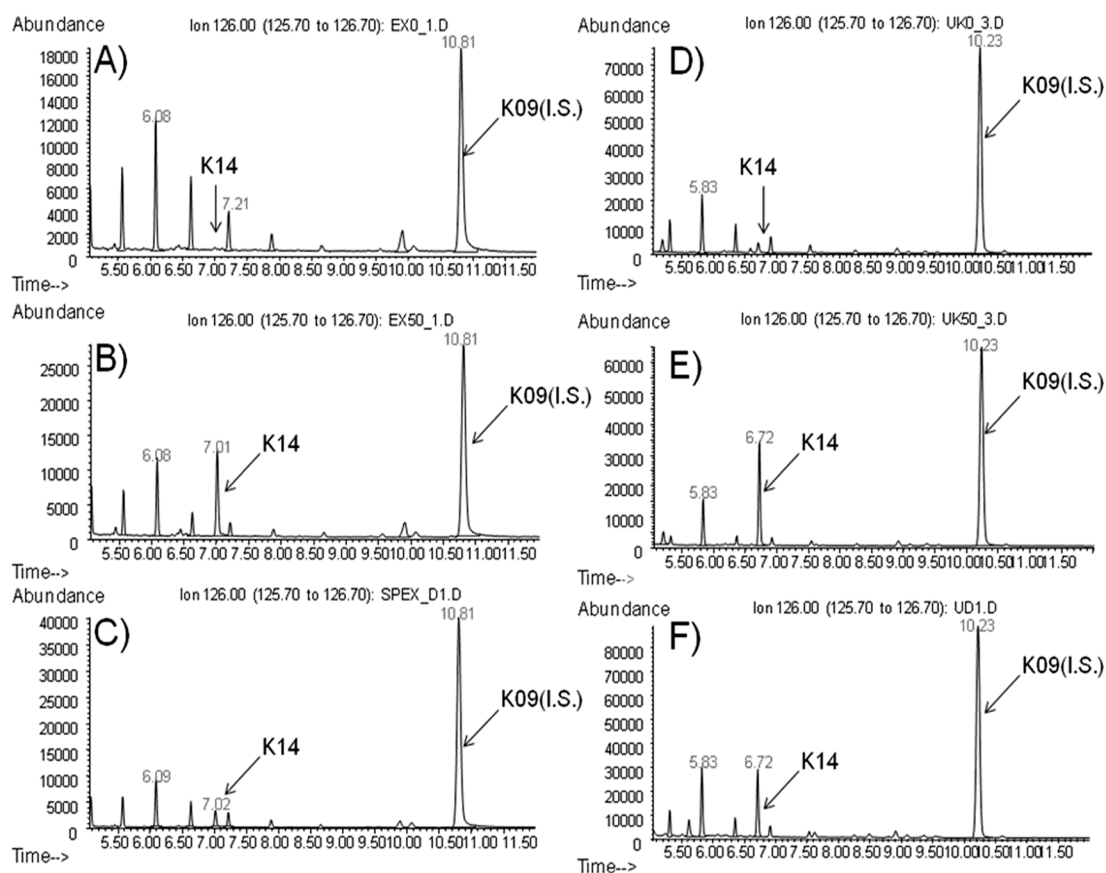


Figure 3—Ion chromatograms of K14 in plasma (A, B, C) and urine (D, E, F): A) K14-free plasma of rats, B) Authentic K14 spiked into K14-free plasma, C) the plasma sample 30 min after oral administration of 20 mg/kg K14 to rats, D) K14-free urine, E) Authentic K14 spiked into urine, and F) the urine sample of the 0-12 hr interval after administration of K14.

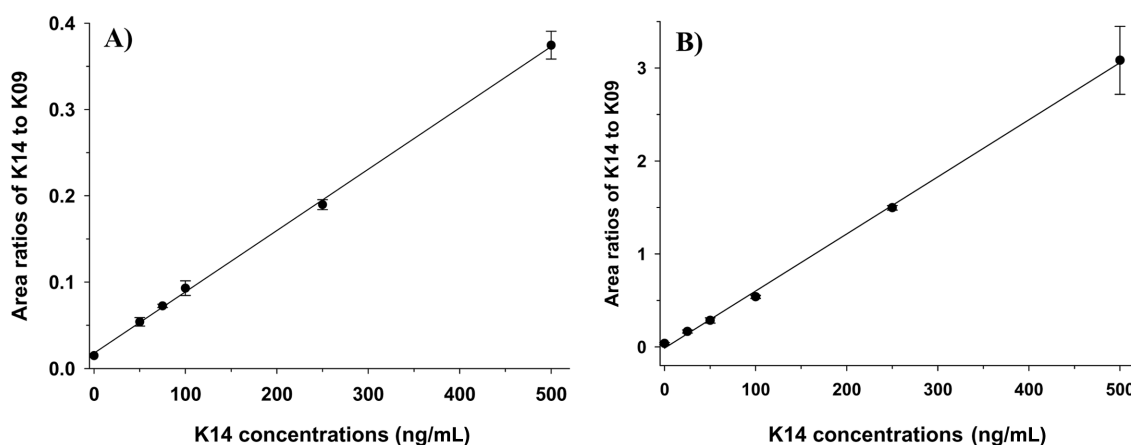


Figure 4—Calibration curves of K14 in plasma (A) and urine (B) of rats. The curve showed high linearity ($r^2 > 0.999$) at concentration ranges (5 to 50 ng/100 μ L plasma; 10-500 ng/mL urine) of K14.

showing that these are good candidate drugs for exerting selective anti-obesity effects (data not shown). Due to newly synthesized chemicals, no analytical methods in plasma and urine were available as well as pharmacokinetic data. In our exper-

iment, the analytical method for the determination of K14 in blood and urine of rats was developed. This method may also be applied to the determination of K09, a synthetic chemical analogue of K14, as indicated in the experiment as internal

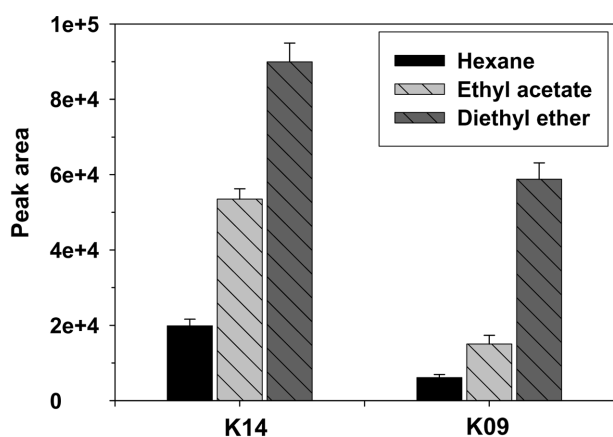


Figure 5—Effects of various solvents on the extraction of K14 and K09.

standard. These data may be useful for further studies of these compounds including absorption and metabolism due to no pharmacokinetic or analytical data available.

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