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Validated LC-MS/MS method for the determination of 3-Hydroxyflavone and its glucuronide in blood and bioequivalent buffers: Application to pharmacokinetic, absorption, and metabolism studies

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Abstract

The purpose of this study is to develop an UPLC-MS/MS method to quantify 3-hydroxy-flavone (3-HF) and its metabolite, 3-hydroxyflavone-glucuronide (3-HFG) from biological samples. A Waters BEH C₈ column was used with acetonitrile/0.1 % formic acid in water as mobile phases. The mass analysis was performed in an API 5500 Qtrap mass spectrometer *via* multiple reaction monitoring (MRM) with positive scan mood. The one-step protein precipitation by acetonitrile was used to extract the analytes from plasma. The results showed that the linear response range was 0.61– 2,500.00 nM for 3-HF and 0.31– 2,500.00 nM for 3-HFG. The intra-day variance is less 16.48 % and accuracy is in 77.70–90.64 % for 3-HF and variance less than 15.86%, accuracy in 85.08–114.70 % for 3-HFG. The inter-day variance is less than 20.23 %, accuracy is in 110.58–114.2 % for 3-HF and variance less than 15.59 %, accuracy in 83.00–89.40% for 3-HFG. The analysis was done within 4.0 min. Only 10 μ L of blood is needed due to the high sensitivity of this method. The validated method was successfully used to pharmacokinetic study in A/J mouse, transport study in the Caco-2 cell culture model, and glucuronidation study using mice liver and intestine microsomes. The applications revealed that this method can be used for 3-HF and 3-HFG analysis in blood as well as in bioequivalent buffers such HBSS and KPI.

Keywords

3-hydroxyflavone; 3-hydroxyflavone-glucuronide; UPLC-MS/MS; Pharmacokinetics; absorption; metabolism

1. Introduction

3-Hydroxyflavone (3-HF, Fig. 1) is a synthesized flavonoid which is not found naturally in plants or other sources such as microorganism and animals. Unlike other flavonoids, there is only one hydroxyl group in the structure of 3-HF. The unique chemical property makes 3-

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HF to serve as a good fluorescent probe to study membranes or intermembrane proteins [1, 2]. For example, It was reported that 3-HF binds with DNA in a groove binding fashion in contrast to the intercalative binding modes in case of other flavonoid probes such as quercetin and fisetin [2, 3]. This binding property renders 3-HF as a tool to study the interaction of small molecules with DNA for exploring the structural and functional features of the biomacromolecules to decipher its biophysical processes. In addition, 3-HF is also used as a chelate agent to deliver metal ions for the treatment of disease. Examples include the attempt of using 3-HF-Zinc complex for the treatment of diabetes [4] and using 3-HF-copper complex for the treatment of tuberculosis [5].

Besides being used as a tool compound, 3-HF also possesses various biological functions. Due to the extremely anti-oxidation property, 3-HF modulates the ERK signaling pathway which regulates a broad range of processes, including cell growth and differentiation, cell survival, apoptosis and embryogenesis [6–8]. Therefore, 3-HF has been viewed as a potent anti-cancer and anti-aging agent [9–11]. In addition, it was reported that the combination of 3-HF and imatinib mesylate resulted in significant apoptotic cell death in imatinib mesylate-resistant leukemia cells [12]. The mechanism study revealed that the above combined treatment resulted in apparent activation of caspases and decrease of the oncoprotein phosphor-Bcr/Abl in leukemia cells [12]. Moreover, 3-HF can also inhibit the ROS (reactive oxygen species) production induced by the UV-irradiation, suggesting 3-HF is useful for improving the *in vitro* development potential of cloned embryos [13].

3-HF belongs to flavonoid which has been shown to possess multiple activities *in vitro* but unsatisfied activity *in vivo*. The major challenge for this class of compounds is poor bioavailability[14]. Since there is one or multiple hydroxyl group(s) in the structure, flavonoid is usually a good substrate of UDP-glucuronosyltransferases (UGTs), which is a classes of phase II metabolism enzymes catalyzing glucuronidation reaction. 3-HF is expected to have similar pharmacokinetic and dispositional characteristics of flavonoids due to its structural similarity. However, the glucuronidation of 3-HF, either *in vitro* or *in vivo*, has not been reported.

All of the above descriptions indicated that 3-HF is being used *in vivo* increasingly. However there is no analytical method for the quantification of 3-HF and its major metabolites in the biological samples. The purpose of this study is to establish a sensitive and reproducible UPLC-MS/MS method for the quantification of 3-HF, as well as its major metabolites 3-HFG, in biological sample and apply this method for absorption, metabolism, and pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

3-HF and biochanin A were bought from Indofine Inc (Hillsborough, NJ, USA). Formic acid, Uridine-5'-diphosphate- β , D-glucuronic acid ester (UDPGA), D-saccharic-1,4-lactone monohydrate, magnesium chloride, and Hanks' balanced salt solution (powder form) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, methanol, and water

(LC-MS grade) were purchased from EMD (Gibbstown, NJ, USA). All other materials were used as received.

2.2. Instruments and conditions

2.2.1 UPLC—UPLC conditions for analyzing 3-HF and its conjugate were: system, Waters AcquityTM with diode array detector (DAD); column, BEH C₈ column (50 × 2.1 mm I.D., 1.7 μm, Waters, Milford, MA, USA); mobile phase A (MPA), 0.1% formic acid in water; mobile phase B (MPB), 100% acetonitrile; gradient, 0–0.5 min, 0–10 % MPB, 0.5–2.5 min, 10–50 % MPB, 2.5–3.0 min, 50–75 % MPB, 3.0–3.5 min, 75–95 % MPB, 3.5–3.7 min, 95–0 % MPB, 3.7–4.0 min, 0% MPB; flow rate, 0.55 ml/min; column temperature, 60 °C; injection volume, 10 μL. Biochanin A was used as the internal standard.

2.2.2 MS—The MS analysis was performed on an API 5500 Qtrap triple quadrupole mass spectrometer (Applied Biosystem/ MDS SCIEX, Foster City, CA, USA) equipped with a TurboIonSprTM source. The concentration of 3-HF and 3-HFG were determined by using MRM (Multiple Reaction Monitoring) scan type in positive mode. The instrument dependent parameters for mass spectrum were set as follows: ionspray voltage, 5.5 kV; ion source temperature, 400 °C; nebulizer gas (gas 1), nitrogen, 20 psi; turbo gas (gas 2), nitrogen 25 psi; curtain gas, nitrogen 30 psi. Unit mass resolution was set in both mass-resolving quadruples Q1 and Q3. Compound-dependent parameters were listed in Table 1.

2.2.3 HPLC—The HPLC conditions for 3-HFG preparation were as follows: an Agilent 1050 running ChemStation software with a 759 A absorbance detector (Applied Biosystem, Foster city, CA); column, Phenomenex Luna C₁₈, 5 μm, 250 × 4.6 mm (Phenomenex, CA); mobile phase A (MPA), acetonitrile; mobile phase B, 0.1% formic acid in water; elution gradient, 0–10 min, 10% MPA, 10–20 min, 10–40% MPA, 20–30 min, 40–90% MPA, 30–35 min, 90% MPA, 35–36 min, 90–10% MPA, 36–40 min, 10% MPA; detect wavelength, 254 nm; flow rate, 1.0 mL/min; injection volume, 200 μL.

2.3. Biosynthesis of 3-HFG

3-HFG was prepared using Caco-2 cell. The culture conditions for growing Caco-2 TC7 cells have been described previously [15]. Briefly, the seeding density was 250,000 cells/mL (2 mL for each well in a 6-well plate), and the cells were grown in a regular growth medium (DMEM supplemented with 10% fetal bovine serum). Caco-2 TC7 cells were fed every other day, and the monolayer was ready for experiments at 11 days after seeding. On 12th day, the Caco-2 TC7 cells were washed with HBSS (pH 7.4, 37 °C) three times. After the HBSS buffer was aspirated, a 3.0 mL solution of 3-HF (10 μM) was added to each monolayer and allowed to incubate for 8 h at 37 °C. The supernatant was then obtained and extracted with methylene chloride twice to remove the parent compound. The resulting sample was then dried in freeze-dry. The residue was dissolved in methanol to remove salt. The methanol solution was then dried under N₂ flow to afford a residue which was reconstituted in 50% methanol for preparation of 3-HFG by HPLC. The purity of the 3-HFG was determined by HPLC (> 98%, based on chromatography peak in UV, 210 and 254 nm).

2.4. Method validation

2.4.1. Calibration curve and LLOD—Calibration curves were prepared the same way as described in section 2.5.4. The linearity of each calibration curve was determined by plotting the peak area ratio of 3-HF and 3-HFG to I.S. in mouse plasma. Least-squares linear regression method ($1/x^2$ weight) was used to determine the slope, intercept and correlation coefficient of linear regression equation. The lower limit of detection (LLOD) was defined based on a signal-to-noise ratio of 10:1.

2.4.2. Precision and accuracy—The “intra-day” and “inter-day” precision and accuracy of the method were determined with QC samples at three different concentrations on the same day or on three different days.

2.4.3. Extraction recovery and matrix effect—The extraction recoveries of 3-HF and 3-HFG were determined by comparing the relative peak areas obtained from blank plasma spiked with analytes and those obtained from water spiked with the same amount of analytes. Matrix effect was determined by comparing the peak areas of blank plasma extracts spikes with analytes and I.S. with those of the standard solutions dried and reconstituted with a mobile phase. These evaluations were performed according to the recommended validation procedures reported by Matuszewski [16].

2.4.4. Stability—The stabilities of 3-HF and 3-HFG in mouse blood were evaluated by analyzing three replicates of QC samples at three different concentrations following storage at 25 °C for 4 h, at –80 °C for 7 days, and after going through three freeze-thaw cycles (–80 °C and 25 °C). The samples were prepared by diluting the stock solution in KPI and then add certain volume of KPI solution into blank blood to final concentrations of 1,250.00, 39.00, 1.22 nM (KPI/blood ratio=1:20 by volume). Stability was expressed as the peak ratio of the mean peak area (triplicate samples) of an analyte at different time points to the mean peak area of the same analyte at time zero multiplied by 100.

2.5. Pharmacokinetics study

2.5.1. Animals—A/J mice (male, 20–25 g, 8 weeks old) were from Harlan Laboratory (Indianapolis, IN) and kept in an environmentally controlled room (temperature: 25 ± 2 °C, humidity: $50 \pm 5\%$, 12 h dark-light cycle) for at least 1 week before the experiments. The mice were fasted overnight before the day of the experiment. The animal protocols used in this study were approved by the University of Houston’s Institutional Animal Care and Uses Committee.

2.5.2. Experimental design—Mice were fasted for 12 h with free access to water prior to the pharmacokinetic study. The drug was administrated at dose of 20 mg/kg in oral suspension vehicle. Briefly, after a mouse was anesthetized with isoflurane gas, 3-HF was gavaged according to the body weight. Afterwards, mouse tail was snipped near the tip of the tail to allow the collection of blood samples. Blood samples (about 15 μ L) were collected in heparinized tubes at 0, 15, 30, 60, 120, 240, 360, 480, 720 and 1440 min, respectively. The blood samples were stored at –80 °C until analysis.

2.5.3. Plasma sample preparation—The blood sample (10 µL) was spiked with 10 µL of KPI and 80 µL of I.S. (Biochanin A in acetonitrile, 0.2 µM). The mixture was vortexed for 1 min. After centrifugation at 15,000 rpm for 15 min, the supernatant was transferred to a new tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 40 µL of 50 % methanol aqueous solution and centrifuged at 15,000 rpm for 15 min. 10 µL of supernatant were injected into the UPLC-MS/MS system for quantitative analysis.

2.5.4. Preparation of standard and quality control samples—Calibration standard samples were prepared in KPI by diluting 3-HF and 3-HFG stock solution (in DMSO) to final concentration of 2,500.00, 1,250.00, 625.00, 312.50, 156.00, 78.00, 39.00, 19.50, 9.75, 4.88, 2.44, 1.22, 0.61, and 0.31 nM, respectively. The calibration standard samples were prepared by spiking the blank mouse blood (10 µL) with above samples in KPI (10 µL) and 80 µL of I.S. The quality control (QC) samples for each compound were prepared at three different concentrations in the same way as the blood samples for calibration were prepared. The QC samples were stored at –80 °C until analysis.

2.5.5. Pharmacokinetics parameter calculation—The pharmacokinetic parameters of 3-HF and 3-HFG were calculated by the non-compartmental method, using Win-Nonlin 3.3 (Pharsight Corporation, Mountain View, California).

2.6. Transport study by Caco-2 cell culture model

2.6.1. Cell culture—Cell culture was according to previously study in our lab [17, 18]. Cells were used at passages 41–49.

2.6.2. Transport experiments in the Caco-2 cell culture model—The experiment protocol and calculation were described in our previously reports. Briefly, 3-HF solution (2.0 µM) was loaded onto the apical or basolateral (donor) side. Five donor samples (500 µL) and five receiver samples (500 µL) were taken at 0, 1, 2, 3, and 4 h followed by the addition of 500 µL of fresh donor solution to the donor side or 500 µL of fresh buffer to the receiver side. The samples were then analyzed by UPLC-MS/MS. The apparent permeability coefficient (P) was determined by the equation

$$P=(dQ/dt)/(A \times C_o)$$

where dQ/dt is the drug permeation rate (µmol/s), A is the surface area of the epithelium (cm²), and C_o is the initial concentration in the donor compartment at time 0 (mM).

2.7 Determination of Glucuronidation rate

The glucuronidation rate of 3-HF (5µM) were determined by measuring the amount of metabolites formed in A/J mouse and small intestinal S9 fraction reaction divided by reaction time (30 min) and protein concentration of S9 fraction (final concentration = 0.0027 mg protein/mL) according to our previously reported procedures [18]. Briefly, the conditions were as follows: (1) mix S9 fraction, magnesium chloride (0.88mM),

saccharolactone (4.4mM), alamethicin 0.022mg/ml), 5 μ M of 3-HF in a 50mM potassium phosphate buffer (pH 7.4), and UDPGA (3.5mM, add last); (2) incubate the mixture (final volume=200 μ L) at 37°C for 1h; (3) end the reaction with the addition of 50 μ L biochanin A (as the internal standard) solution in acetonitrile. The samples were vortexed for 30s, centrifuged at 15,000 rpm for 10min, and 10 μ L of supernatant was injected into UPLC–MS/MS system for analysis.

3. Results and discussion

3.1 Optimization of the UPLC-MS condition

The method was developed by optimizing both the UPLC and the MS/MS conditions to obtain the best possible sensitivity and peak shape. Methanol, acetonitrile, 2.5 mM ammonia acetate (pH = 7.6), 0.1–0.5 % formic acid, and 100% water were tested as potential mobile phases. Both C₁₈ and C₈ columns (Acquity, Waters, 2.1 $\times$ 50 mm, 1.7 μ m) were evaluated as solid phases. According to the intensity and the peak shape, acetonitrile and 0.1% formic acid were selected as mobile phases and C₈ column was selected as solid phase. In addition, in order to obtain a sharp and symmetrical peak, the column temperature was set up at 60°C and the flow rate was 0.55 ml/min.

For the MS condition, both positive and negative scan modes were evaluated. According to the intensity of the analytes, positive scan was selected. Multiple reaction monitoring (MRM) scan type was used to improve the specificity. The compounds and instrument dependent parameters were optimized by tuning these three analytes separately. The results are shown in Table 1.

3.2 Method validation

3.2.1 Linearity and lower limit of detection (LLOD) and lower limit of quantification (LLOQ)—The method validation was conducted in blank mouse blood. The standard curves were linear in the concentration range of 2,500.00–0.61 nM for 3-HF and 2,500.00–0.31 nM for 3-HFG ($R^2 > 0.99$). The lower limit of detection (LLOD) was 0.31 nM for both of the analytes. LLOQ for 3-HF and 3-FHG were 0.61 and 0.31 nM respectively.

3.2.2 Accuracy and precision—Accuracy, intra-day and inter-day precision were determined by measuring six replicates of QC samples at three concentration levels in mouse blood. The precision and accuracy were shown in Table 2. These results demonstrated that the precision and accuracy values were in the acceptance range.

3.2.3 Recovery, matrix effect and stability—The mean extraction recoveries determined using three replicates of QC samples at three concentration levels (the same concentrations as QC sample) in mouse blood were shown in Table 3. Methanol was used to extract 3-HF and 3-HFG in blood samples. The result showed the recoveries were not less than 75 % for these two analytes at low, medium, and high concentrations.

As for testing the matrix effects that may impact UPLC-MS analysis, the relative peak areas of these two analytes after spiking evaporated blood samples at three concentration levels

were comparable to similarly prepared aqueous standard solutions. The results were reported in Table 3, which suggested that there was no measurable matrix effect observed.

The stabilities of 3-HF and 3-HFG in mouse blood were evaluated by analyzing three replicates of QC samples at three different concentrations (1,250.00, 39.00, 1.22 nM) following storage at 25 °C for 4 h, at – 80 °C for 7 days, and after going through three freeze-thaw cycles (–80 °C and 25 °C). All the samples displayed no less than 85% recoveries after various stability tests (Table 3).

3.3. Identification of metabolite from in vitro and in vivo samples

After globe LC-MS search, 3-HFG was identified as the major metabolites in pharmacokinetic study and the transport study using the Caco-2 cell culture mode by comparing the retention times with that of standard 3-HFG obtained from biosynthesis, which were further confirmed by MS/MS spectral data. In Caco-2 cell culture experiment and pharmacokinetic study, one major metabolite peak was observed at 2.10 min (Fig 2A). In MS analysis, the peak at 2.10 min showed molecular weight of m/z 415 $[M+H]^+$, which released a fragment of m/z 239 $[M-GluA+H]^+$ in MS^2 experiment (Fig 3B). Since there is only one hydroxyl group in the structure, the conjugate position for glucuronidation was assigned for position-3. Therefore the peak was identified as 3-HFG.

3.6 Application in mouse p.o. pharmacokinetics, Caco-2 experiment, and phase II metabolism studies

The validated method was employed to study the pharmacokinetic behaviors of 3-HF in A/J mice. The mean plasma concentration-time curves of 3-HF and its metabolites (3-HFG) after oral administration (20 mg/kg dose) were shown in Fig 4. For 3-HF, the C_{max} was 218 nM, the T_{max} was 105 minutes, and the $AUC_{0-\infty}$ was 2396.89 hr* nM (Table 4). 3-HFG was detected as the major metabolites. The T_{max} of 3-HF was 330 minutes and the C_{max} was 1800 nM. The $AUC_{0-\infty}$ values were 48219.22 hr* nmol/L for 3-HF. The observation suggested that glucuronide is the major metabolism pathway of 3-HF after oral administration.

The validated method was also used to determine the transport and metabolites excretion of 3-HF in the Caco-2 cell culture model. The vectorial transport result showed that the apical to basolateral permeability (P_{ab}) of 3-HF ($4.56 \pm 1.09 \times 10^{-6}$ cm/sec) was the similar to its basolateral to apical permeability (P_{ba} , $4.51 \pm 1.46 \times 10^{-6}$ cm/sec) (Fig 5A). The results showed that 3-HF had good permeation across the Caco-2 cell monolayer at both directions. Since the P_{ab} is similar as P_{ba} , most likely, 3-HF follows passive diffusion transport mechanism. 3-HFG was identified as the major metabolite in the transport study. For the excretion of 3-HFG, the basolateral side excretion is always higher than that of apical side no matter where the 3-HF was loaded (Fig 5B,C). Most likely, efflux transporter(s) (e.g., MRP3) in the basolateral side was involved in the excretion of 3-HFG. Further study, such as using chemical inhibitor or siRNA, is needed to identify the involved transporters.

The validated method was also used to measure samples derived from in vitro metabolism studies of 3-HF. The results revealed that the glucuronidation rate of 3-HF (5.0 μ M) in

mouse liver and small intestinal S9 fraction were 2.06 ± 0.05 and 2.75 ± 0.05 nmol/min/mg protein, respectively (Fig 6). This rate indicated a fast metabolism and the result could explain why extremely high level of 3-HFG was detected in the PK study.

4. Conclusion

A rapid, sensitive and specific UPLC-MS/MS method had been developed and validated for the determination of 3-HF and 3-HFG in plasma as well as bioequivalent buffers. The main advantages of this method are: 1) small blood volume (10 μ L); 2) rapid analysis (4.0 min); 3) simple sample preparation procedure and good recovery; (4) minor matrix effect. Application of this analysis method to p.o. pharmacokinetic study showed that 3-HF is extensively metabolized *in vivo* with glucuronides as the major metabolic pathway. Since 3-HF and its metabolite can be analyzed simultaneously, each mouse can be used to derive a complete pharmacokinetic profile. This method is also valuable for human clinical study because it should allow higher sensitivity than reported here because large volume of blood sample is usually available in human, which may be used to concentrate the analytes before analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

UPLC	ultra-performance liquid chromatography
IS	internal standard
DP	declustering potential
CE	collision energy
CXP	collision cell exit potential
AUC	area under the curve
QC	quality control
LLOQ	lower limit of quantification
3-HF	3-hydroxyflavone
3-HFG	3-hydroxyflavone-glucuronide
UDPGA	Uridine-5'-diphosphate- β , D-glucuronic acid ester
MPA	mobile phase A
MPB	mobile phase B
KPI	Potassium Phosphate" buffer

HBSS

Hank's Balanced Salt Solution

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Figure 1.
Chemical structures of 3-HF, 3-HFG, and biochanin A (I.S.)

Figure 2.

Representative MRM chromatograms of 3-HF, 3-HFG, and biochanin A (I.S.) in a mouse blood sample. A, blank blood spiked with 3-HF (2.4nM), 3-HFG (2.4nM), and I.S.; B, blank blood spiked with I.S. only. The retention time of 3-HF, 3-HFG, and biochanin A were 2.10 (3-HFG), 2.42 (I.S.), and 2.53 (3-HF) min respectively.

Figure 3.

The MS/MS spectrum of 3-HF (A) and 3-HFG (B). The results showed the molecular ion 415 $[M+H]^+$ and its MS² fragment at 239 $[M+H-Glu]^+$ for 3-HFG.

Figure 4.

Plasma concentrations of 3-HF (square) and 3-HFG (diamond) after p.o. administration of 20 mg/kg 3-HF in A/J mice (n=5). Blood sample (10 μ L) was spiked into KPI (10 μ L), and I.S. (80 μ L, biochanin A in acetonitrile, 0.2 μ M). The mixture was vortexed and centrifuged for 15 min at 15,000rpm. The supernatant was taken out and reconstituted in 40 μ L of 50 % methanol and centrifuged for another 15 min at the same speed. Then 10 μ L of supernatant were injected into the UPLC-MS/MS system. Each point is average of five determinations and the error bars are standard deviations of the mean.

Figure 5.

Transport of 3-HF in the Caco-2 cell culture model (A) and time course of conjugate excretion in the Caco-2 cell culture model (B and C). The buffer used in both donor and receiver sides was HBSS (pH = 7.4). The donor side concentrations of 3-HF were always 2 μ M either from apical to basolateral or basolateral to apical directions. The incubation time points were 60, 120, 180 and 240 min. The experiment was performed at 37°C. Each data point is the average of three determinations. Bars are standard deviations of the mean.

Figure 6.
The glucuronidation rate of 3-HF (5μM) by A/J mouse liver and small intestinal S9 fraction reaction. Each data point is the average of three determinations, and the error bar is the standard deviation of the mean.

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Table 1

Compound-dependent parameters in UPLC-MS analysis

Compound	Q1(<i>m/z</i>)	Q3(<i>m/z</i>)	Dwell time (ms)	DP (V)	EP(V)	CE(V)	CXP(V)
3-HFG	415	239	150	171	10	21	16
3-HF	239	165	150	163	10	42	11
I.S.	285	151	150	187	10	33	9

Table 2

Intra-day and inter-day precision and accuracy of 3-HF and 3-HFG.

Analytes	Linear range (nM)	Concentration (nM)	Intra-day		Inter-day	
			Accuracy (bias, %)	Precession (CV %)	Accuracy (bias, %)	Precession (CV %)
3-HF	2,500.00-0.60	1.22	92.66	13.20	92.55	13.38
		39.00	85.30	6.42	102.1	4.91
		1,250.00	84.58	4.58	106.09	11.24
3-HFG	2,500.00-0.31	1.22	105.64	15.65	97.95	15.37
		39.00	92.90	13.27	106.47	9.41
		1,250.00	108.68	14.82	106.15	5.96

Table 3

Recovery, matrix effect, and stability of 3-HF and 3-HFG at high, medium, and low concentrations.

Compound	Concentration (nM)	Recovery		Matrix Effect*		Room temperature (25°C)		Stability†	
		Mean (%)	SD			2 h	4h	–80°C 7 days	freeze-thaw 3 cycles
3-HF	1.22	105.46	7.37	119.22		106.38	88.31	100.36	82.60
	39.00	89.60	10.06	91.65		86.23	99.03	86.31	87.56
	1,250.00	110.81	11.65	77.98		89.57	84.41	86.97	110.8
3-HFG	1.22	95.00	9.67	115.13		83.71	98.55	89.52	84.40
	39.00	77.87	6.59	92.62		99.91	94.85	84.71	94.48
	1,250.00	112.51	8.63	98.29		84.29	87.41	90.23	109.70

* Matrix effect expressed as the ratio of the mean peak area of an analyte spiked post-extraction to the mean peak area of the same analyte standards multiplied by 100.

† Stability expressed as the peak ratio of the mean peak area of an analyte at different time points to the mean peak area of the same analyte at time zero multiplied by 100.

Table 4

Pharmacokinetics parameters of 3-HF and 3-HFG after p.o. administration at 20 mg/kg dose (n = 5).

	<i>T</i> _{max} (min)	<i>C</i> _{max} (nM)	<i>AUC</i> _{0-∞} (h*nM)	<i>t</i> _{1/2} (min)	<i>K</i> _e (1/h)	<i>V</i> _{ss} (ml/kg)	<i>CL</i> (ml/h/kg)
3-HF	105.00 ± 99.50	218.00 ± 156.43	2396.89 ± 936.19	884.77 ± 596.88	0.006 ± 0.003	0.15 ± 0.04	0.01 ± 0.001
3-HFG	330.00 ± 266.08	1800.00 ± 495.58	48219.22 ± 19257.84	-	-	-	-