Published in final edited form as:

Biomed Chromatogr. 2018 February ; 32(2): . doi:10.1002/bmc.4064.

Sensitive liquid chromatography/tandem mass spectrometry method for the determination of two novel highly lipophilic anti-cancer drug candidates in rat plasma and tissues

Shirin Hooshfar¹, Michael R. Linzey¹, Daqing Wu², Lajos Gera³, Kenza Mamouni², Xin Li², Yanhua Chen², Yang Yang², Oluwasegun Olorunyolemi¹, and Michael G. Bartlett¹

¹Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA 30602-2352, United States

²Department of Biochemistry and Molecular Biology, Augusta University, Augusta, GA 30912, United States

³Department of Biochemistry and Molecular Genetics, University of Colorado Denver, Anschutz Medical Campus, Aurora, CO 80045, United States

Abstract

A simple and sensitive liquid chromatography/electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) method was developed and validated for determination of two highly lipophilic anti-cancer drug candidates, LG1980, and GH501, in rat plasma and tissues (liver, kidney and femur bones).

LG1980 and GH501 were extracted from rat plasma and tissue homogenates using liquid–liquid extraction. The method provided a linear range of 1.0–200.0 ng/mL for GH501 in plasma and LG1980 in plasma and liver. For both analytes in other tissue homogenates the linear range was 2.0–400.0 ng/mL. The method was validated with precision within 15% relative standard deviation (RSD), accuracy within 15% relative error (RE) and a consistent recovery. This method has been successfully applied in two preclinical studies for LG1980 and GH501 to determine their concentrations in rat plasma, liver, kidney and bone over 24 h after intravenous injection of compounds.

Keywords

Highly lipophilic drug; LC–MS/MS; Plasma; Tissue; Bioanalysis

1. Introduction

Following heart disease, cancer is the second most common cause of human suffering and death in the United States (US) (Narang and Desai, 2009; Siegel *et al.*, 2016, 2017). According to a recent annual report from the American Cancer Society, in 2017, an

estimated 1,688,780 new cases of cancer will be diagnosed in the US and 600,920 people will die from the disease (almost 1 in 4 deaths) (Siegel *et al.*, 2017).

Lung, breast, prostate, and colorectum cancers account for 46% of all cancer deaths in men and women (Siegel *et al.*, 2017). Although the 5-year relative survival rate for patients at an earlier stage of all cancers has increased over the past 3 decades and despite the recent approval of numerous anticancer drugs, most of the diagnosed patients with distant metastasis cancers do not overcome the disease (Ali *et al.*, 2012; López-Lázaro, 2015; Miller *et al.*, 2016; S. Zhang *et al.*, 2013). The five-year survival rates for people diagnosed with distant lung, breast, prostate, and colorectum cancers are 4, 26, 29 and 14 % respectively (Miller *et al.*, 2016; Siegel *et al.*, 2017). Therefore, there is an urgent need to develop new anticancer agents with better anti-tumor, anti-metastatic activities and lower side effects to overcome therapeutic resistance and improve the survival rate.

Flurbiprofen analogs are known as broad spectrum anticancer small molecules that are particularly useful for the treatment of lung, pancreatic and head and neck cancers (Gera, Chan, Hodges, *et al.*, 2013). GH501 is a new highly potent flurbiprofen analog with the growth inhibitory activity against a large variety of human tumor cell lines including breast, colon, central nervous system (CNS), leukemia, melanoma, non-small cell lung (NSCL), ovarian, prostate and renal cancers (Gera, Chan, Bunn, *et al.*, 2013; Gera, Chan, Hodges, *et al.*, 2013; Gera *et al.*, 2015). The average 50% growth inhibition concentration (GI₅₀) value for the 60 cell lines from these 9 cancer types was 498 nM for GH501 (Gera *et al.*, 2015). A good understanding of pharmacokinetic and toxicokinetic properties are essential to evaluate the performance of new drug candidates in biological systems. Therefore, it is necessary to investigate the pharmacokinetics of GH501 as a potential new drug candidate with extensive anticancer activity, during preclinical studies in animal models.

Amino-bisphosphonate derivatives are another group of novel small-molecules, designed for targeting bone metastatic prostate cancer. These compounds have been shown to effectively inhibit tumor growth in bone, reduce prostate-specific antigens and improve bone structure in animal studies (Gera *et al.*, 2008; Seo *et al.*, 2008; Sh. Zhang *et al.*, 2016). LG1980 is a representative of this class of molecules that selectively induces apoptosis in aggressive prostate cancer cells. This compound is also a candidate for preclinical studies to determine its toxicity and pharmacokinetics in biological systems. The chemical structures of GH501 and LG1980 are shown in Fig. 1.

It is worth mentioning that flurbiprofen analogs or amino-bisphosphonate derivatives contain molecules with very lipophilic structures (Gera, Chan, Hodges, *et al.*, 2013; Gera *et al.*, 2008). Both GH501 and LG1980 are highly lipophilic compounds with the experimental partition coefficient (LogP) values of 6.84 and 7.83, respectively. These values are much higher than the logPs for several highly lipophilic approved drugs such as ritonavir (LogP: 6), cinnarizine (LogP: 5.8), simvastatin (LogP: 4.7) and paclitaxel (LogP: 3.3) (Li *et al.*, 2013; Lu *et al.*, 2015; "National Center for Biotechnology Information. CID=36314," accessed Feb. 23, 2017; "National Center for Biotechnology Information. CID=54454," accessed Feb. 23, 2017; "National Center for Biotechnology Information. CID=392622," accessed Feb. 23, 2017; "National Center for Biotechnology Information. CID=1547484," accessed Feb. 23,

2017). As far as we know halofantrine is the only small molecule approved drug with higher LogP (8.6) than our analytes ("National Center for Biotechnology Information. CID=37393," accessed Feb. 23, 2017). This lipophilic nature presents a challenge toward the development of a reliable and sensitive analytical method and creation of effective sample preparation for extraction and sample clean-up in biological matrices.

To the best of our knowledge, no analytical method has been reported for the determination of flurbiprofen analogs or amino-bisphosphonate derivatives in biological samples. In this study we develop a sensitive and selective liquid chromatography tandem mass spectrometry (LC–MS/MS) method for determination of GH501 and LG1980 in rat plasma and tissues (Liver, kidney and femur bone). This bioanalytical method was validated according to the current FDA guidelines and successfully applied to preclinical studies of these compounds ("Guidance for Industry, Bioanalytical Method Validation, " 2001). Additionally, this method could be applicable for quantification of other analogs belonging to these two groups with minor modification. The developed sample preparation method could be a very good template for effective extraction and sample clean-up for other highly lipophilic compounds from complex matrices such as rat plasma, liver, kidney and femur bone.

2. 2. Material and methods

2.1. Chemicals and reagents

GH501, LG1980, GH101 (Internal standard, IS) and BKM1972 (IS) were synthesized according to the procedure described by Gera et al (Gera, Chan, Hodges, *et al.*, 2013; Gera *et al.*, 2008). The chemical structures of these analytes are shown in Fig. 1. Sodium hydroxide, ammonium formate, formic acid, acetonitrile (ACN), methyl tert-butyl ether (MTBE), water and Spin-X® centrifuge filters were obtained from Sigma–Aldrich (St. Louis, MO). Sodium phosphate, dibasic (Na₂HPO₄) was purchased from J.T. Baker (Center Valley, PA). All solvents were LC-MS grade and all reagents were analytical grade. Lo-bind microcentrifuge tubes were obtained from Eppendorf® (Hauppauge, NY). Male Sprague Dawley rat plasma (with sodium EDTA), liver, kidney and femur bone were purchased from Bioreclamation IVT (Westbury, NY).

2.2. Instrumentation

An Agilent 1100 binary pump HPLC system (Santa Clara, CA) interfaced to a Waters Micromass Quattro Micro triple quadrupole mass spectrometer with an electrospray ionization (ESI) source (Milford, MA) was operated for LC–MS/MS analysis. Masslynx 4.1 software by Waters (Beverly, MA) was used for instrument control and data processing.

A tissue homogenizer (Polytron® pcu-2) and probe (Kinematica Polytron, PTA-10s, 10mm stator 7mm rotor) from Brinkmann Instruments (Westbury, NY) were used for homogenization of tissues. A Labconco CentriVap Complete Vacuum Concentrator (Kansas City, MO) was utilized to dry samples.

2.3. LC-MS/MS conditions

The chromatographic separation was achieved using a Waters Atlantis dC-18 (2.1 × 30 mm, 3 µm) column coupled with a Phenomenex SecurityGuard C-18 guard column (4.0 mm × 2.0 mm). The column temperature was controlled at 25 °C. Mobile phase A was 10 mM ammonium formate/0.1% formic acid in water and mobile phase B was ACN. The injection volume was 30 µL and the flow rate was 0.3 mL/min. The autosampler injection needle was washed with ACN after each injection. The gradient condition for GH501 was: (time/minute, % mobile phase B): (0, 30), (8, 80), (9, 30), (15, 30) and for LG1980 was as follows: (time/minute, % mobile phase B): (0, 45), (0.1, 95), (4, 95), (5, 45), (10, 45). Samples were analyzed by the mass spectrometer in positive ion ESI mode. Nitrogen was used as the desolvation gas at a flow rate of 500 L/h for GH501 and 400 L/h for LG1980. For GH501, the cone gas flow was set to 30 L/h. For both compounds the desolvation temperature was 500 °C and the source temperature was 120 °C. Argon was used as the collision gas and the collision cell pressure was 2.5×10^{-3} m bar for GH501 and 5.5×10^{-3} m for LG1980. A summary of the cone voltages, collision energies, and precursor and product ions of the analytes is presented in Table 1.

2.4. Solutions and standards

Primary stock solutions of GH501, GH101 (IS), LG1980 and BKM1972 (IS) were prepared by dissolving exact amounts of compounds in ACN to obtain a final concentrations of 1.0 mg/ml. The secondary stock solutions of all analytes were prepared by diluting the primary stock solutions to a concentration of 10 µg/mL.

Standard working solutions of LG1980 were obtained by serial dilutions to concentrations of 10.0, 20.0, 40.0, 100.0, 200.0, 400.0, 1000.0 and 2000.0 ng/mL. Quality control (QC) working solutions were prepared at concentrations of 30.0, 300.0, and 1500.0 ng/mL. The BKM1972 (IS) working solution was prepared at 200.0 ng/mL. These working solution were used for making calibration curves and QC samples in plasma and liver homogenate. The same set of working standard solutions were made for GH501 and its IS GH101, using the previously-mentioned method. These standards were consumed for making the calibration curve and QC samples only in plasma.

Another set of calibration working solutions of LG1980 were prepared at concentrations of 20.0, 40.0, 100.0, 200.0, 400.0, 1000.0, 2000.0 and 400 ng/mL by serial dilutions from the secondary stock solutions. The QC working solutions were 50, 500 and 3500 ng/mL. The BKM1972 (IS) working solution was 400 ng/mL. These set of working solution were used for making calibration curves and QC samples in kidney and bone homogenate. The same set of standards were prepared for GH501 and GH101 (IS). These standards were spiked in liver, kidney and bone homogenate to make the calibration curves and QC samples.

All dilutions were made with ACN. Stock solutions were kept at -20 °C when not in use and replaced every 3 months. Fresh working solutions were prepared for each day of analysis or validation.

2.5. Spiked samples

10 μL of standard or QC working solution was spiked into 100 μL of plasma or tissue homogenate to generate the corresponding standard or QC sample. This yields calibration standard concentrations of 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, 100.0 and 200.0 ng/mL for GH501 in plasma and for LG1980 in plasma and liver homogenate. The QC samples were 3, 30.0 and 150.0 ng/mL.

The final concentrations of the calibration standards were 2.0, 4.0, 10.0, 20.0, 40.0, 100.0, 200.0 and 400.0 ng/mL for LG1980 in kidney and bone homogenate and for GH501 in kidney, bone and liver homogenate. The QC samples were 5, 50.0 and 350.0 ng/mL.

The blank femur bone sample was weighed and then crushed using a mortar and pestle. The small particles of the prepared bone sample were transferred to a glass vials. The physiological saline (0.89%, w/v NaCl) with the ratio of 1:3 w/w for bone to saline, was added to the sample. In the next step, the tissue homogenizer was used to further break up the bone particles and make a cloudy, white suspension.

Liver and kidney homogenates were prepared using physiological saline in a ratio of 1 part tissue to 2 parts saline by using the tissue homogenizer.

2.6. Sample preparation

To each 100 μL rat plasma or tissue homogenate (liver, kidney and bone), 10 μL IS working solution, 100 μL 0.5 M Na_2HPO_4 (pH 11.5) and 1.2 mL MTBE were added. Additionally, 10 μL sodium fluoride (0.64 M) was added to each sample in order to inhibit non-specific enzyme activities. All samples were kept on ice during the sample preparation. The mixture was vortexed for 10 min, then centrifuged at $21,130 \times g$ at 5°C for 10 min. 1.0 mL organic supernatant was transferred and evaporated to complete dryness in a vacuum concentrator at 45°C for 10 min.

For plasma samples and LG1980 samples from liver or bone homogenate, the sample was reconstituted with 100 μL of 60:40 mobile phase A: mobile phase B and vortex mixed for 5 min. The sample was then centrifuged at $21,130 \times g$ at 5°C for 5 min. For the plasma sample, 90 μL of supernatant was transferred for injection. For the LG1980 sample obtained from liver or bone homogenate the supernatant was filtered before injection using a Spin-X[®] centrifuge filter (pore size: 0.22 μm , centrifuged at $3,381 \times g$ for 30 s).

In the case of GH501 samples from all tissue homogenates (liver, kidney and bone) and the LG1980 sample from kidney homogenate, after drying, the pellet was extracted with 500 μL of ACN and vortex mixed for 10 min. The sample was then centrifuged at $21,130 \times g$ at 5°C for 10 min. The supernatant was evaporated to complete dryness in a vacuum concentrator at 45°C . Final reconstitution was performed with 100 μL mobile phase A: mobile phase B (60:40) and vortex mixed for 5 min. The sample was then centrifuged at $21,130 \times g$ at 5°C for 5 min. The supernatant was filtered before injection using a Spin-X[®] centrifuge filter (pore size: 0.22 μm , centrifuged at $3,381 \times g$ for 30 s).

2.7. Method validation

The bioanalytical method was validated in accordance with current U.S. FDA guidelines including selectivity, linearity, intra- and interday precision and accuracy, recovery and stability ("Guidance for Industry, Bioanalytical Method Validation," 2001). Selectivity (n = 6) was tested by comparing the chromatograms of analytes in blank plasma, or in tissue homogenate (liver, kidney and bone), with analytes in the same matrix at the lower limit of quantitation (LLOQ).

The linearity was tested by the use of spiked standards over the range from 1.0–200.0 ng/mL in plasma (GH501 and LG1980) and liver (LG1980), and 2.0–400.0 ng/mL for both compounds in the rest of the tissue homogenates (GH501: kidney, liver and bone. LG1980: kidney and bone). Calibration curves were constructed from peak area ratios between the analyte and IS, using 1/x weighted linear regression.

The intra-day (n = 5) and inter-day (n = 15) precision and accuracy were assessed by analyzing QC samples at 1.0 (LLOQ), 3.0, 30.0 and 150 ng/mL for the plasma (both analytes) and liver homogenate (only LG1980) and 2.0 (LLOQ), 5.0, 50.0 and 350.0 ng/mL for both compounds in other tissue homogenates (GH501: kidney, liver and bone. LG1980: kidney and bone).

Recovery (n = 3), including matrix effect (ME), relative recovery (RR) and absolute recovery (AR) in plasma and tissue homogenates (liver kidney and bone) were calculated from peak areas of spiked samples, post-preparation spiked samples and standard solutions at QC levels.

Autosampler stability, bench-top stability, and freeze/thaw stability in all matrices were tested at the low QC and high QC of the method.

2.8. Animal study

For each compound (LG1980 or GH501), three male Sprague-Dawley rats (approximately 200 g; Envigo Rms Inc., Madison, WI) were housed in a temperature controlled room (25 °C), maintained on a 12:12 h normal light–dark cycle with free access to water and food until used for two separate animal studies. All procedures employed in this study were reviewed and approved by the Augusta University Institutional Animal Care and Use Committee and are consistent with AAALAC guidelines. Measures were taken to minimize pain and discomfort in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996.

The dose of 10 mg/kg (GH501) and 20 mg/kg (LG1980) were prepared in dimethyl sulfoxide (DMSO). The compound (GH501 or LG1980) was injected through the tail vein of the rats, and the blood samples were collected from facial veins. Blood samples were collected at 15 min, 30 min, 2 h, 6 h, 12 h and 24 h post dosing. These samples were immediately centrifuged at $2,000 \times g$ at 4 °C for 10 min and the plasma was separated. Liver, kidney and two femur bones of the sacrificed animals were removed and washed with normal saline. Tissue samples were homogenized in the same way as blank tissues (tissue to

normal saline ratio: 1/2 for liver and kidney, tissue to normal saline ratio: 1/3 for bone). All samples were kept frozen at -80°C until analysis.

3. Results and discussion

3.1. Method development

In order to develop a sensitive and accurate method for quantification of GH501 or LG1980 within the biological samples, different factors and parameters were optimized for both the high-performance liquid chromatography (HPLC) and the tandem mass spectrometry (MS/MS).

The method development in this study started with LG1980. The final method with minor modifications was applied for determination of GH501 in plasma and tissues.

Both LG1980 and GH501 are very lipophilic compounds, making them good targets for liquid-liquid extraction (LLE). These compounds are also weakly basic (See Fig. 1), thus a higher pH facilitates their neutralization in the aqueous phase, promotes distribution to the organic phase and increases the extraction efficiency. Therefore, 100 μL 0.5 M Na_2HPO_4 (pH 11.5) was added to the sample to provide good extraction efficiency at a moderately basic pH.

Extraction using MTBE and diisopropyl ether have been shown to provide good results with significantly higher analyte recoveries for lipophilic analytes in biological samples (Bylda *et al.*, 2014). LLE with diisopropyl ether produced a very clean extract, but the LLOQ of the method for LG1980 in the rat plasma was around 10 ng/ml. One step LLE with MTBE made the method 10-fold more sensitive (LLOQ: 1 ng/mL). It seems, due to the hydrophobic nature of this solvent, during the extraction step, the analyte can distribute to the organic phase much easier.

In tissue homogenates, final samples contained high amounts of biological residues, most likely lipids. Extraction using MTBE, especially at basic pHs can show a high recovery for phospholipids in biological samples (Bylda *et al.*, 2014). These co-extracted biological materials caused high chromatographic background and retention time shift. Therefore, we decided to filter liver and bone samples using Spin-X[®] centrifuge filters, before injection. In this way, the LLOQ for LG1980 in liver and bone homogenate were 1 and 2 ng/mL, respectively.

In kidney homogenate after the drying step, samples contained a high amount of lipid residue. Reconstitution of this greasy pellet produced a suspension of small particles. Even after centrifugation, the sample was cloudy and contained an oily upper layer. Due to the liquid nature of this layer, using Spin-X[®] centrifuge filters for the sample clean-up, wasn't useful in this step. Although the total lipid percentage of rat liver (4.7 %, w/w of tissue) and kidney (3.5 %, w/w of tissue) is similar, the different composition of phospholipids in these two tissues is believed to be the reason for this phenomenon (Connellan and Masters, 1965; Jüngst *et al.*, 1988).

To eliminate this problem, we performed additional sample clean-up by adding another extraction step. With this procedure, after LLE, separation and drying the organic phase, the pellet was extracted with 500 μ L of ACN for 10 min. Then the sample was centrifuged, filtered, dried (45 °C, 45 min) and dissolved in 100 μ L of the initial mobile phase before analysis. By using this method, the LLOQ for LG1980 in kidney homogenate was 2 ng/mL.

For extraction of GH501 from plasma and kidney homogenate the same method of sample preparation for LG1980 in these two matrices was used.

However, the described one step LLE method that was used for extraction of LG1980 from liver and bone homogenate wasn't suitable for extraction of GH501 from these tissues. By using this method, the LLOQ of GH501 for liver and bone homogenate was around 10 ng/mL.

In order to make the method more sensitive, we decided to try the described extraction step with adding 500 μ L of ACN to our sample preparation method for GH501 in bone and liver homogenates. This final modified LLE clean-up made our method 5-fold more sensitive for these two tissues (LLOQ: 2 ng/mL). As mentioned before, in tissue homogenates, the sample after drying step contained a high amount of the lipid residue. Due to the lipophilic nature of these two compounds, reconstitution with mobile phase cannot extract most of the analytes from this lipophilic pellet. LG1980 has a stronger MS signal than GH501 in biological samples or even neat solutions. Therefore, unlike GH501, this extracted portion of LG1980 was enough to give us the LLOQ of 1–2 ng/mL for liver and bone homogenate and further extraction with ACN for this analyte was not necessary.

GH101 and BKM1972 were selected as IS for GH501 and LG1980, respectively. GH101 is a flurbiprofen analog, and differs from GH501 in only a methyl group on the α -carbon and a fluorine atom on the phenyl group. BKM1972 is one of the amino-bisphosphonate derivatives and has the most similar chemical structure to LG1980 in this group of compounds.

For the sensitive detection of analytes, the triple quadrupole mass spectrometer was set to operate under unit mass resolution. Since the analytes are weak bases, positive ion mode was used. With the constant infusion of a neat solution of each analyte (10 μ g/mL in ACN) at a flow rate of 50 μ L/min, the ion source parameters were optimized to achieve the strongest intensity of MS signal. The detection of analytes and ISs were conducted using multiple reaction monitoring (MRM) functions, providing high sensitivity and selectivity. Collision energy and cone voltages were also optimized with direct infusion of individual standard solutions. The most abundant fragment ion for each compound was selected for the monitored ion-transition in MRM mode. The determined ion transitions were 734 $\rightarrow$ 207 for LG1980, 745 $\rightarrow$ 196 for BKM1972, 607 $\rightarrow$ 84 for GH501 and 575 $\rightarrow$ 84 GH101.

It is noteworthy that transitions of m/z 607 $\rightarrow$ 196 and 575 $\rightarrow$ 196 were used as a confirmation for GH501 and GH101, respectively. For BKM1972, the in source generated fragment ion at m/z 745 was more abundant than the protonated molecule ion $[M + H]^+$ at m/z 790. This fragment ion resulting from the loss of one ethoxide moiety and was then used for subsequent MS/MS experiments monitoring the transition from m/z 745 $\rightarrow$ 196.

3.2. Selectivity

Representative chromatograms obtained from blank biological matrices and spiked LLOQ standards are shown in Figs. 2 and 3.

The chromatograms were compared between the same type of matrix and no interference from endogenous substances or other components were observed at the retention times of GH501 or LG1980 in blank samples.

3.3. Linearity and sensitivity

Calibration curves were made from peak area ratios between analytes and the IS using 1/x weighted linear regression. Table 2 shows the calibration curves for each day of validation for both analytes.

The R^2 values are over 0.99 for analytes in all matrices, demonstrating the good linearity of the method when determining GH501 or LG1980 in plasma and tissue homogenates (liver, kidney and bone) within the tested range.

The sensitivity of the method was defined by the LLOQ, which was considered to be the lowest concentration that could be measured with a signal-to-noise ratio (S/N) of 10 within 20% precision and accuracy. LLOQ for LG1980 was 1 ng/mL in plasma and liver homogenate and 2 ng/ml in kidney and bone homogenate. LLOQ for GH501 was 1 ng/ml for plasma and 2 ng/ml in other tissue homogenates (Figs. 2 and 3).

3.4. Precision and accuracy

Accuracy was calculated by comparing the concentration of spiked samples to each nominal concentration ($(\text{nominal concentration} - \text{measured concentration} / \text{nominal concentration}) \times 100$). Precision was reported as percentage relative standard deviation (% RSD) among measured concentrations.

Precision and accuracy were calculated for LLOQ and QC samples of analytes in all matrices, as shown in Table 3 and 4, which met the FDA requirements of less than 20 % for LLOQ and less than 15 % for QC samples.

3.5. Recovery

AR, RR and ME were tested for the analytes at the three QC concentrations ($n = 3$) in all matrices, as shown in Tables 5 and 6.

For each concentration of analytes in all matrices, three spiked samples, three post-preparation spiked samples and three neat solutions were prepared. The post-preparation spiked samples were made by spiking standard solutions into blank matrices processed by the same sample preparation. The neat standards for each concentration were prepared by diluting GH501 or LG1980 working solutions with the initial mobile phase.

The AR was represented by the ratio of peak areas between spiked samples and the average of standard solutions at the same concentration. The RR was represented by the ratio of peak areas between spiked samples and the average of corresponding post-preparation spiked

samples. ME was represented by the ratio of peak areas between post-preparation spiked samples and the average of corresponding standard solutions. A ratio over 100% demonstrates an enhancing ME, a ratio below 100% demonstrates a suppressing ME, and the percentage of enhancement or suppression is calculated as the absolute difference from 100%.

AR and RR for both analytes in all matrices, are consistent, precise and reproducible at 3 concentrations (Tables 5 and 6). A lower AR and RR were observed in tissue homogenates compared to plasma for both compounds. This is mainly due to the lower extraction efficiency in liver, kidney and bone homogenates, as a result of interference from lipids and/or protein impurities in tissues.

Both ion suppression and enhancement were observed for GH501 and LG1980 in the matrices. The co-extraction of lipid-based or protein-based impurities could have happened during the LLE by MTBE. These biological residues can compete with the analytes during ESI and cause variable suppression or enhancement in MS response of analytes.

3.6. Stability

To validate autosampler stability, two sets of spiked samples ($n = 3$) at high QC and low QC were prepared in plasma and tissue homogenates. The first set was analyzed immediately and used as a time zero control, and the second set was placed on the autosampler for a while and then analyzed. For both analytes in all matrices, samples were stable on the autosampler at room temperature for 12 h. (Tables 7 and 8).

Two sets of low QCs and high QCs ($n = 3$) were prepared for the evaluation of bench top stability of samples. One set was immediately extracted and analyzed, and the second set was left at ambient temperature before extraction. Even though sodium fluoride had been added to plasma and tissue samples to inhibit the metabolism of analytes by endogenous enzymes, bench top stability didn't pass for durations longer than 30 and 60 minutes for LG1980 and GH501, respectively. However, when the samples were kept at 4°C, GH501 samples were stable for more than 3 h and LG1980 samples were stable for 1 h (Tables 7 and 8). It seems these compounds are not stable in biological samples for a long time. Therefore, samples should be kept on ice the entire time during extraction while the final samples can be left in a non-refrigerated autosampler for LC-MS/MS analysis.

For evaluation of Freeze-thaw stability, one set of high QCs and low QCs ($n = 3$) were analyzed without freezing as a time zero control. Three other sets were frozen at -80°C , thawed the next day, and one set of them was analyzed. This was repeated for two more freeze-thaw cycles for 2 additional days, and the responses were compared with respect to those of the initial unfrozen samples. The results showed that any observed difference in the measured amounts of analytes after freeze-thaw was within the experimental error of the method and freezing did not have an effect on the sample stability (Tables 7 and 8).

3.7. Application

In order to demonstrate the applicability of this method in a practical biological situation, the analytical method was successfully applied to the quantification of analytes in rat plasma, liver, kidney and bone samples during two separate pharmacokinetic studies.

The concentration/time profile for GH501 and LG1980 extracted from rat plasma following the intravenous (IV) dosing (GH501: 10 mg/kg, LG1980: 20 mg/kg) of rats over a 24 h time-period are presented in Fig. 4. The concentrations of analytes in liver, kidney and bone samples, 24 h post IV injection are shown in table 9. The representative chromatograms from the analysis of real plasma and tissue samples are demonstrated in Fig. 5. In all samples the concentrations of GH501 and LG1980 were within the linear range.

Assuming 1 g brain is equivalent to 1 mL of plasma, all tissue-to-plasma ratios for GH501 were higher than 20.3 (Table 9). These high ratios indicate a high permeability and distribution of GH501 in liver, kidney and bone. The high volume of distribution (V_d) for GH501 (87.71 L) also supports this conclusion. The pharmacokinetic parameters for GH501 and LG1980 were calculated using plasma concentrations and are shown in Table 10. For LG1980 the tissue-to-plasma ratios are between 0.2–0.8 (Table 9). Also, the clearance (CL) for this compound (8.27 L/min) is much higher than the CL of GH501 (1.67 L/min). However, these ratios still showed that LG1980 could substantially distribute in to these tissues. Nevertheless, this data should be taken with caution because of the limited number of animals ($n=3$), and further pharmacokinetic studies would be required to make a final conclusion.

4. Conclusion

For the first time, a reliable and sensitive LC-MS/MS method has been successfully developed and applied to the determination of two highly lipophilic anti-cancer new drug candidates in rat plasma and tissues (liver, kidney and femur bone).

The bioanalytical method exhibited good accuracy and precision over the linear range of 1.0–200.0 ng/mL in rat plasma (for both GH501 and LG1980) and liver homogenate (only for LG1980) and 2.0–400.0 ng/mL (GH501 and LG1980) in other tissue homogenates. The LLOQ of the method was 1.0 ng/mL for both analytes in plasma and LG1980 in liver homogenate. The LLOQ for both compounds in other tissue homogenates was 2.0 ng/mL.

In this method, LG1980 and GH501 were extracted from biological samples using liquid–liquid extraction. Due to a low sample volume (100 μ L), the method can be easily applied to preclinical studies requiring multiple samples in small animals. The validated method has been successfully applied in preclinical studies, following IV administration of analytes to rats.

Acknowledgments

This project was supported by grants from National Cancer Institute (grant numbers: 1R41CA186498-01A1 and 1R41CA206725-01A1), Research Alliance Ventures, University of Georgia/Augusta University Cancer Research Initiative Award, and Georgia Cancer Center Startup Fund (D. Wu).

References

- Ali R, Mirza Z, Ashraf GM, Kamal MA, Ansari SA, Damanhour GA, Abuzenadah AM, Chaudhary AG, Sheikh IA. New anticancer agents: recent developments in tumor therapy. *Anticancer Research*. 2012; 32:2999–3005. [PubMed: 22753764]
- Bylda C, Thiele R, Kobold U, Volmer DA. Recent advances in sample preparation techniques to overcome difficulties encountered during quantitative analysis of small molecules from biofluids using LC-MS/MS. *Analyst*. 2014; 139:2265–2276. DOI: 10.1039/C4AN00094C [PubMed: 24633191]
- Connellan J, Masters C. Fatty acid components of rat-tissue lipids. *Biochemical Journal*. 1965; 94:81. [PubMed: 14342254]
- Gera, L., Chan, DC., Bunn, PA., Hodges, RS. Paper presented at the The Twenty-Third American and the Sixth International Peptide Symposium. Hilton Waikoloa Village, HI, U.S.A: 2013. Design and Synthesis of Small Molecule Peptidomimetics as Broad Spectrum Anticancer Drug Candidates; p. 192-193.
- Gera, L., Chan, DC., Hodges, RS., Bunn, PA. Flurbiprofen analogs and methods of use in treating cancer: Google Patents, US. 8,575,170 B2. 2013.
- Gera, L., Stewart, JM., Chung, LW., Wu, D. Compositions and methods for treating bone cancer: Google Patents. WO2008124756 A1. 2008.
- Gera, L., Tentler, J., Eckhardt, SG., Jiang, Z., D'Alessandro, A., Hodges, RS. Paper presented at the The Twenty-Fourth American Peptide Symposium. Orlando, FL, U.S.A: 2015. Development of Novel Broad Spectrum Anticancer Small Molecule Peptidomimetics with Nanomolar Activity; p. 241-244.
- Guidance for Industry. Bioanalytical Method Validation. 2001
- Jüngst D, Blumenstingl J, Caselmann WH. Isolation and characterization of proteolipids from rat liver (LSP) and kidney (KSP). *Lipids*. 1988; 23:410–414. [PubMed: 3412118]
- Li P, Albrecht BJ, Yan X, Gao M, Weng HR, Bartlett MG. A rapid analytical method for the quantification of paclitaxel in rat plasma and brain tissue by high-performance liquid chromatography and tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 2013; 27:2127–2134. DOI: 10.1002/rcm.6671 [PubMed: 23996385]
- López-Lázaro M. Two preclinical tests to evaluate anticancer activity and to help validate drug candidates for clinical trials. *Oncoscience*. 2015; 2:91–98. [PubMed: 25859551]
- Lu Y, Qiu Y, Qi J, Feng M, Ju D, Wu W. Biomimetic reassembled chylomicrons as novel association model for the prediction of lymphatic transportation of highly lipophilic drugs via the oral route. *International Journal of Pharmaceutics*. 2015; 483:69–76. doi:<http://dx.doi.org/10.1016/j.ijpharm.2015.02.017>. [PubMed: 25681731]
- Miller KD, Siegel RL, Lin CC, Mariotto AB, Kramer JL, Rowland JH, Stein KD, Alteri R, Jemal A. Cancer treatment and survivorship statistics, 2016. *CA: A Cancer Journal for Clinicians*. 2016; 66:271–289. DOI: 10.3322/caac.21349 [PubMed: 27253694]
- Narang, AS., Desai, DS. *Pharmaceutical perspectives of cancer therapeutics*. Springer; 2009. Anticancer drug development; p. 49-92.
- National Center for Biotechnology Information. [accessed Feb. 23, 2017] CID=36314. (Publication no. <https://pubchem.ncbi.nlm.nih.gov/compound/36314>). from PubChem Compound Database
- National Center for Biotechnology Information. [accessed Feb. 23, 2017] CID=37393. (Publication no. <https://pubchem.ncbi.nlm.nih.gov/compound/37393>). from PubChem Compound Database
- National Center for Biotechnology Information. [accessed Feb. 23, 2017] CID=54454. (Publication no. <https://pubchem.ncbi.nlm.nih.gov/compound/54454>). from PubChem Compound Database
- National Center for Biotechnology Information. [accessed Feb. 23, 2017] CID=392622. (Publication no. <https://pubchem.ncbi.nlm.nih.gov/compound/392622>). from PubChem Compound Database
- National Center for Biotechnology Information. [accessed Feb. 23, 2017] CID=1547484. (Publication no. <https://pubchem.ncbi.nlm.nih.gov/compound/1547484>). from PubChem Compound Database
- Seo SI, Gera L, Zhau HE, Qian WP, Iqbal S, Johnson NA, Zhang S, Zayzafoon M, Stewart J, Wang R. BKM1740, an acyl-tyrosine bisphosphonate amide derivative, inhibits the bone metastatic growth

of human prostate cancer cells by inducing apoptosis. *Clinical Cancer Research*. 2008; 14:6198–6206. [PubMed: 18829499]

Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA: A Cancer Journal for Clinicians*. 2016; 66:7–30. DOI: 10.3322/caac.21332 [PubMed: 26742998]

Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. *CA: A Cancer Journal for Clinicians*. 2017; 67:7–30. DOI: 10.3322/caac.21387 [PubMed: 28055103]

Zhang S, Gera L, Mamouni K, Li X, Chen Z, Kucuk O, Wu D. Inhibition of skeletal growth of human prostate cancer by the combination of docetaxel and BKM1644: an aminobisphosphonate derivative. *Oncotarget*. 2016; 7:27489. [PubMed: 27050371]

Zhang S, Wang Y, Chen Z, Kim S, Iqbal S, Chi A, Ritenour C, Wang YA, Kucuk O, Wu D. Genistein enhances the efficacy of cabazitaxel chemotherapy in metastatic castration-resistant prostate cancer cells. *Prostate*. 2013; 73:1681–1689. DOI: 10.1002/pros.22705 [PubMed: 23999913]

GH501 **GH101** **LG1980** **BKM1972****Fig. 1.**

Chemical structure of GH501 (MW = 605.78 g/mol, LogP = 6.86), GH101 (MW = 573.77 g/mol, LogP = 6.13), LG1980 (MW = 732.75 g/mol, LogP = 7.83) and BKM1972 (MW = 789.62 g/mol, LogP = 8.48).

Fig. 2.

Representative chromatograms of LG1980 and BKM1972 (IS (A) blank plasma, (B) plasma at LLOQ of method (1.0 ng/mL), (C) blank liver homogenate, (D) liver homogenate at LLOQ of method (1.0 ng/mL), (E) blank kidney, (F) kidney at LLOQ of method (2.0 ng/mL), (G) blank bone homogenate and (H) kidney homogenate at LLOQ of method (2.0 ng/mL). Retention times for analytes are shown in minutes.

Fig. 3.

Representative chromatograms of GH501 and GH101(IS) in (A) blank plasma, (B) plasma at LLOQ of method (1.0 ng/mL), (C) blank liver homogenate, (D) liver homogenate at LLOQ of method (2.0 ng/mL), (E) blank kidney, (F) kidney at LLOQ of method (2.0 ng/mL), (G) blank bone homogenate and (H) bone homogenate at LLOQ of method (2.0 ng/mL). Retention times for analytes are shown in minutes.

Fig. 4. Application of the bioanalytical method: (A) time-course of LG1980 extracted from rat plasma ($n = 3$), (B) time-course of GH501 extracted from rat plasma ($n = 3$), following the intravenous (IV) dosing (GH501: 10 mg/kg, LG1980: 20 mg/kg) of rats over a 24 h time-period.

Fig. 5. Representative chromatograms of: (A) LG1980 extracted from rat plasma, liver, kidney and bone samples, and (B) GH501 extracted from rat plasma, liver, kidney and bone samples, 24 h after intravenous (IV) dosing (GH501: 10 mg/kg, LG1980: 20 mg/kg) of rats. Retention times for analytes are shown in minutes.

Table 1

Optimized ESI (+) mass spectrometric conditions for multiple reaction monitoring (MRM) of compounds.

Compound	Precursor ion (m/z)	Proiduct ion (m/z)	Collision energy (eV)	Cone voltage (V)
GH501	607	84	55	30
GH101 (IS)	575	84	45	30
LG1980	734	207	35	15
BKM 1972 (IS)	745	196	45	30

Table 2

Calibration curves for LG1980 and GH501 in plasma and tissue homogenates (n = 3).

Analyte	Matrix	Slope	Intercept	R ²
LG1980	Plasma	0.4921 ± 0.0790	0.1802 ± 0.2405	0.9930 ± 0.0042
	Liver homogenate	0.3519 ± 0.1107	0.2034 ± 0.1137	0.9972 ± 0.0024
	Kidney homogenate	0.1538 ± 0.0233	-0.1589 ± 0.1416	0.9952 ± 0.0020
	Bone homogenate	0.1472 ± 0.0241	-0.0743 ± 0.0658	0.9923 ± 0.0015
GH501	Plasma	0.9718 ± 0.0383	0.7063 ± 0.1735	0.9956 ± 0.0005
	Liver homogenate	1.1318 ± 0.0253	2.4578 ± 0.3775	0.9977 ± 0.0019
	Kidney homogenate	1.6572 ± 0.5441	1.7307 ± 1.6840	0.9978 ± 0.0005
	Bone homogenate	1.3549 ± 0.0066	2.2345 ± 0.2648	0.9990 ± 0.0009

Table 3

The intra- and inter-batch precision (%RSD) and accuracy (%RE) of LG1980 extracted from rat plasma and tissue homogenates.

Matrix	Nominal conc. (ng/mL)	Intra-day			Inter-day		
		Measured conc. $\pm$ S.D (ng/mL)	RSD (%)	RE (%)	Measured conc. $\pm$ S.D (ng/mL)	RSD (%)	RE (%)
Plasma	1.0 (LLOQ)	1.03 $\pm$ 0.13	12.28	-2.5	1.01 $\pm$ 0.12	12.10	-0.91
	3.0 (low QC)	3.10 $\pm$ 0.33	10.70	8.58	2.89 $\pm$ 0.34	11.86	-3.33
	30.0 (mid QC)	30.18 $\pm$ 1.23	4.07	3.30	28.54 $\pm$ 1.93	6.78	-0.60
	150.0 (high QC)	138.68 $\pm$ 7.88	5.68	7.58	137.39 $\pm$ 10.98	7.99	7.55
Liver homogenate	1.0 (LLOQ)	1.00 $\pm$ 0.14	14.14	0.00	0.93 $\pm$ 0.15	16.37	6.67
	3.0 (low QC)	2.98 $\pm$ 0.36	12.19	8.66	2.93 $\pm$ 0.34	11.43	0.67
	30.0 (mid QC)	31.76 $\pm$ 1.19	3.76	6.16	29.39 $\pm$ 1.92	6.54	-5.87
	150.0 (high QC)	168.10 $\pm$ 4.25	2.53	12.04	150.97 $\pm$ 13.41	8.88	-12.07
Kidney homogenate	2.0 (LLOQ)	2.03 $\pm$ 0.31	15.02	-1.67	1.98 $\pm$ 0.25	12.52	0.83
	5.0 (low QC)	5.04 $\pm$ 0.45	8.94	-0.80	5.37 $\pm$ 0.39	7.27	-7.33
	50.0 (mid QC)	53.98 $\pm$ 3.88	7.19	-7.95	54.11 $\pm$ 4.98	9.21	-8.23
	350.0 (high QC)	373.13 $\pm$ 18.06	4.84	-6.61	377.91 $\pm$ 17.17	4.54	-7.97
Bone homogenate	2.0 (LLOQ)	2.13 $\pm$ 0.17	7.97	-6.67	2.15 $\pm$ 0.15	6.98	-7.5
	5.0 (low QC)	5.54 $\pm$ 0.51	9.26	-10.80	5.34 $\pm$ 0.57	10.66	-6.80
	50.0 (mid QC)	53.00 $\pm$ 2.37	4.47	-6.00	52.73 $\pm$ 2.46	4.67	-5.45
	350.0 (high QC)	370.98 $\pm$ 15.07	4.06	-5.99	362.25 $\pm$ 22.92	6.33	-3.50

Table 4

The intra- and inter-batch precision (%RSD) and accuracy (%RE) of GH501 extracted from rat plasma and tissue homogenates.

Matrix	Nominal conc. (ng/mL)	Intra-day			Inter-day		
		Measured conc. $\pm$ S.D (ng/mL)	RSD (%)	RE (%)	Measured conc. $\pm$ S.D (ng/mL)	RSD (%)	RE (%)
Plasma	1.0 (LLOQ)	0.90 $\pm$ 0.17	19.25	10.00	0.93 $\pm$ 0.15	16.22	7.50
	3.0 (low QC)	2.72 $\pm$ 0.15	5.45	9.33	2.57 $\pm$ 0.23	8.87	14.22
	30.0 (mid QC)	31.16 $\pm$ 1.17	3.76	-3.87	29.27 $\pm$ 1.71	5.85	2.43
	150.0 (high QC)	145.30 $\pm$ 2.15	1.48	3.13	139.59 $\pm$ 6.11	4.38	6.94
Liver homogenate	2.0 (LLOQ)	1.77 $\pm$ 0.12	6.54	11.67	1.93 $\pm$ 0.33	17.16	3.75
	5.0 (low QC)	4.73 $\pm$ 0.61	12.86	5.50	4.80 $\pm$ 0.44	9.25	4.00
	50.0 (mid QC)	51.63 $\pm$ 3.52	6.83	-3.25	53.02 $\pm$ 7.71	14.53	-6.04
	150.0 (high QC)	361.83 $\pm$ 13.43	3.71	-3.38	371.30 $\pm$ 15.85	4.27	-6.09
Kidney homogenate	2.0 (LLOQ)	2.07 $\pm$ 0.15	7.39	-3.33	1.98 $\pm$ 0.22	11.23	1.25
	5.0 (low QC)	5.00 $\pm$ 0.67	13.47	0.00	5.34 $\pm$ 0.66	12.32	-6.80
	50.0 (mid QC)	52.05 $\pm$ 2.46	4.73	-4.10	51.97 $\pm$ 2.07	3.98	-3.94
	350.0 (high QC)	356.75 $\pm$ 14.71	4.12	-1.93	348.07 $\pm$ 17.59	5.05	0.55
Bone homogenate	2.0 (LLOQ)	1.90 $\pm$ 0.20	10.53	5.00	1.88 $\pm$ 0.17	9.11	6.25
	5.0 (low QC)	5.14 $\pm$ 0.62	12.08	-2.80	5.22 $\pm$ 0.53	10.11	-4.40
	50.0 (mid QC)	54.29 $\pm$ 1.42	2.62	-8.58	54.39 $\pm$ 4.44	8.17	-8.78
	350.0 (high QC)	325.11 $\pm$ 8.32	2.56	7.11	327.15 $\pm$ 13.95	4.27	6.53

Table 5

Absolute recovery (%AR, n = 3), relative recovery (%RR, n = 3) and matrix effect (%ME, n = 3) of the method for LG1980.

Matrix	Conc. (ng/mL)	AR (%)	RR (%)	ME (%)	Type of effect
Plasma	3.0	71.51 ± 5.62	71.51 ± 5.62	96.03 ± 4.17	3.97 Suppression
	30.0	80.37 ± 0.65	68.41 ± 0.55	117.47 ± 3.46	17.47 Enhancement
	150.0	73.72 ± 2.08	68.97 ± 1.94	106.90 ± 3.33	6.90 Enhancement
Liver homogenate	3.0	35.53 ± 6.41	37.07 ± 6.69	95.86 ± 6.48	4.14 Suppression
	30.0	41.86 ± 4.89	41.69 ± 3.87	103.36 ± 5.69	3.36 Enhancement
	150.0	42.07 ± 3.09	50.16 ± 3.68	92.89 ± 1.57	7.11 Suppression
Kidney homogenate	5.0	24.68 ± 3.57	24.01 ± 3.48	102.77 ± 8.60	2.77 Enhancement
	50.0	26.87 ± 2.38	23.80 ± 2.11	112.91 ± 5.20	12.91 Enhancement
	350.0	28.15 ± 7.05	23.38 ± 5.86	120.40 ± 8.61	20.40 Enhancement
Bone homogenate	5.0	44.93 ± 6.42	35.17 ± 4.14	140.20 ± 6.99	40.20 Enhancement
	50.0	42.11 ± 5.96	30.83 ± 1.23	150.72 ± 5.45	50.72 Enhancement
	350.0	40.40 ± 3.83	34.29 ± 2.66	121.25 ± 5.15	21.25 Enhancement

Table 6

Absolute recovery (%AR, n = 3), relative recovery (%RR, n = 3) and matrix effect (%ME, n = 3) of the method for GH501.

Matrix	Conc. (ng/mL)	AR (%)	RR (%)	ME (%)	Type of effect
Plasma	3.0	81.305 ± 8.54	70.44 ± 7.40	115.42 ± 3.40	Enhancement
	30.0	77.118 ± 4.66	66.17 ± 4.00	116.55 ± 7.01	Enhancement
	150.0	73.361 ± 2.08	70.48 ± 1.99	104.08 ± 8.07	Enhancement
Liver homogenate	5.0	49.69 ± 2.25	76.40 ± 3.46	65.04 ± 3.36	Suppression
	50.0	43.87 ± 4.37	51.53 ± 5.13	85.13 ± 5.98	Suppression
	350.0	62.91 ± 4.51	62.36 ± 4.47	100.89 ± 3.32	Enhancement
Kidney homogenate	5.0	35.50 ± 3.72	56.95 ± 5.97	62.34 ± 9.21	Suppression
	50.0	32.75 ± 3.17	66.86 ± 6.46	48.98 ± 3.16	Suppression
	350.0	31.00 ± 6.33	67.15 ± 4.53	50.16 ± 5.73	Suppression
Bone homogenate	5.0	66.69 ± 9.03	65.87 ± 8.92	101.25 ± 9.62	Enhancement
	50.0	71.66 ± 5.08	63.59 ± 4.51	112.69 ± 2.28	Enhancement
	350.0	79.43 ± 4.10	69.86 ± 3.61	113.69 ± 3.80	Enhancement

Table 7

Stability testing of LG1980 in rat plasma and tissue homogenates (n=3).

Matrix	Stability	Nominal conc. (ng/mL)	Measured conc. ± S.D (ng/mL)	RSD (%)	RE (%)
Plasma	Autosampler stability	3.0	3.020 ± 0.249	8.245	6.740
		150.0	139.620 ± 5.388	3.859	6.920
	Bench top stability	3.0	2.726 ± 0.065	2.379	9.133
		150.0	127.875 ± 4.864	3.804	14.750
	Freeze-thaw stability	3.0	2.914 ± 0.404	13.847	11.393
		150.0	137.973 ± 6.427	4.658	8.009
Liver homogenate	Autosampler stability	3.0	2.85 ± 0.53	18.73	15.00
		150.0	146.58 ± 13.01	8.87	7.08
	Bench top stability	3.0	3.20 ± 0.32	10.00	9.33
		150.0	141.96 ± 13.02	9.17	7.16
	Freeze-thaw stability	3.0	2.90 ± 0.59	20.46	17.33
		150.0	139.49 ± 19.84	14.22	12.64
Kidney homogenate	Autosampler stability	5	5.63 ± 0.17	3.04	-12.50
		350	385.33 ± 2.58	0.67	-10.09
	Bench top stability	5	5.18 ± 0.61	11.85	-3.50
		350	372.43 ± 18.21	4.89	-6.41
	Freeze-thaw stability	5	4.82 ± 0.68	14.10	3.67
		350	335.68 ± 25.25	7.52	4.09
Bone homogenate	Autosampler stability	5	5.28 ± 0.19	3.59	-5.50
		350	374.78 ± 11.32	3.02	-7.08
	Bench top stability	5	5.30 ± 0.28	5.34	-6.00
		350	309.33 ± 23.58	7.62	11.62
	Freeze-thaw stability	5	5.61 ± 0.33	5.83	-12.25
		350	347.32 ± 25.35	7.30	0.77

Table 8

Stability testing of GH501 in rat plasma and tissue homogenates (n=3).

Matrix	Stability	Nominal conc. (ng/mL)	Measured conc. ± S.D (ng/mL)	RSD (%)	RE (%)
Plasma	Autosampler stability	3.0	2.83 ± 0.15	5.39	5.56
		150.0	135.3 ± 3.49	2.58	9.80
	Bench top stability	3.0	2.73 ± 0.32	11.76	9.00
		150.0	150.17 ± 3.87	2.57	-0.11
	Freeze-thaw stability	3.0	2.93 ± 0.32	10.82	2.33
		150.0	151.02 ± 9.27	6.14	-0.68
Liver homogenate	Autosampler stability	5	5.40 ± 0.78	14.46	-8.00
		350	388.53 ± 13.25	3.41	-11.01
	Bench top stability	5	4.95 ± 0.64	12.86	1.00
		350	396.73 ± 8.19	2.06	-13.35
	Freeze-thaw stability	5	4.69 ± 0.69	14.78	6.20
		350	322.65 ± 9.75	3.02	7.81
Kidney homogenate	Autosampler stability	5	5.70 ± 0.17	3.04	-14.00
		350	339.93 ± 12.99	3.82	2.88
	Bench top stability	5	4.83 ± 0.25	5.09	3.37
		350	319.89 ± 9.57	2.99	8.60
	Freeze-thaw stability	5	4.79 ± 0.40	8.46	4.30
		350	336.80 ± 28.50	8.46	3.77
Bone homogenate	Autosampler stability	5	5.27 ± 0.64	12.21	-5.33
		350	324.80 ± 10.75	3.31	7.2
	Bench top stability	5	4.83 ± 0.25	5.09	3.43
		350	319.89 ± 9.57	2.99	3.29
	Freeze-thaw stability	5	4.79 ± 0.40	8.46	4.3
		350	336.80 ± 28.50	8.46	3.77

Table 9

Concentrations and tissue to plasma ratios, for LG1980 and GH501 in plasma, liver, kidney and bone, 24 h after a single IV injection.

Analyte	Plasma conc. (ng/mL)	Liver conc. (ng/g)	Liver to plasma ratio	Kidney con. (ng/g)	Kidney to plasma ratio	Bone conc. (ng/g)	Bone to plasma ratio
GH501	45.97 ± 11.24	935.85 ± 46.46	20.36	1912.50 ± 497.66	41.60	1349.00 ± 112.29	29.35
LG1980	35.87 ± 12.37	27.9 ± 3.1	0.78	26.13 ± 10.5	0.73	7.20 ± 1.20	0.20

Table 10

Elimination rate constant (K), volume of distribution (Vd), half-life ($T_{1/2}$) and clearance (CL) calculated from plasma concentration of GH501 and LG1980.

Analyte	K	V _d (L)	T _{1/2} (min)	CL (L/min)
GH501	0.0189 ± 0.0053	87.71 ± 20.76	38.97 ± 11.04	1.66 ± 0.39
LG1980	0.0430 ± 0.0076	192.30 ± 6.73	15.86 ± 2.61	8.27 ± 2.89