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A liquid chromatography with tandem mass spectrometry method for simultaneous determination of UTL-5g and its metabolites in human plasma

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Abstract

UTL-5g is a novel small-molecule TNF- α inhibitor under investigation as both a chemoprotective and radioprotective agent. Animal studies showed that pretreatment of UTL-5g protected kidney, liver, and platelets from cisplatin-induced toxicity. In addition, UTL-5g reduced liver and lung injuries induced by radiation *in vivo*. Although a number of preclinical studies have been conducted, a validated bioanalytical method for UTL-5g in human plasma has not been published. In this work, a sensitive and reproducible reverse-phase liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) assay was developed and validated for the determination of UTL-5g and its metabolites, 5-methylisoxazole-3-carboxylic acid (ISOX) and 2,4-dichloroaniline (DCA), in human plasma. The method involves a simple methanol precipitation step followed by injection of the supernatant onto a Waters 2695 HPLC system coupled with a Waters Quattro Micro™ triple quadrupole mass spectrometer. Chromatographic separation was accomplished using a Waters Nova-Pak C18 column maintained at 30 °C, running at gradient mode with mobile phase consisting of 0.1% formic acid in water and 0.1% formic acid in methanol at a flow rate of 0.2 mL/min. The analytes were monitored under positive electrospray ionization (ESI). Quantitation of these compounds in plasma was linear from 0.05 to 10 μ M. The lower limit of quantitation (LLOQ) was 0.05, 0.1, and 0.2 μ M for UTL-5g, ISOX and DCA, respectively. The accuracy and intra- and inter-day precisions were within the generally accepted criteria for bioanalytical method (<15%). This method provides a practical tool to measure and characterize the plasma concentration-time profiles for UTL-5g and its metabolites, ISOX and DCA.

Keywords

UTL-5g; Bioanalytical method validation; LC-MS/MS

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1. Introduction

UTL-5g (Fig. 1) is a novel small-molecule TNF- α inhibitor under preclinical development as a potential agent for both chemoprotection and radioprotection. Preclinical animal studies showed that pretreatment of UTL-5g significantly lowered AST/ALT and creatinine/BUN levels elevated by cisplatin; pretreatment of UTL-5g also increased the platelet count reduced by cisplatin [1,2]. These chemoprotective effects of UTL-5g were >3 times more efficient than those of amifostine based on molecular concentration [2]. UTL-5g also increased the tolerability of cisplatin in mice and increased the survival rates of mice treated with high doses of cisplatin treatment [1]; this observation further corroborated its chemoprotective effects against the toxicity of cisplatin. As to radioprotection, UTL-5g reduced the acute liver injury induced by radiation *in vivo* [3]; in addition, UTL-5g lowered levels of both TGF- β in blood and TNF- α in lung, which were elevated by lung irradiation, but did not affect tumor-response to radiation [4]. These results indicate that UTL-5g is an active agent that reduces a high level of toxicity indicator caused by cisplatin or radiation. In terms of toxicity, UTL-5g has a low acute toxicity (IC₅₀ value >2000 mg/kg) [2], slightly better than that of the widely prescribed broad-spectrum chemoprotector/radioprotector, amifostine (LD₅₀ ~ 709–2025 mg/kg) [5]. Furthermore, a 7-day repeat dose toxicity study of UTL-5g under GLP showed a result of No Observed (Adverse) Effect Level, NO(A)EL (Not yet published). Therefore, there is a great potential for UTL-5g to continue its path in the preclinical development.

Although a number of preclinical studies have been conducted, including the identification of the enzymatic products of UTL-5g under esterase [6], a validated bioanalytical method for UTL-5g and its metabolites in human plasma has not been established. In this work, the *in vitro* metabolites of UTL-5g in human plasma, 5-methylisoxazole-3-carboxylic acid (ISOX) and 2,4-dichloroaniline (DCA), were identified for the first time. Furthermore, this is the first report detailing a simple sample preparation from human plasma and a reverse-phase liquid chromatography coupled to a tandem mass spectrometry (LC–MS/MS) for a rapid analysis of UTL-5g, and its two metabolites, ISOX and DCA. Because UTL-5g is both an active drug and a prodrug based on our previous studies [7], it was of great interest to quantify both UTL-5g and its metabolites.

2. Materials and methods

2.1. Chemicals and Reagents

UTL-5g (Lot#1182-MEM-3D, Purity>99%) was synthesized at Kalexsyn Medicinal Chemistry (Kalamazoo, MI). ISOX (Lot 10121557 CAS# 3405-77-4) and DCA (Lot 10128795 CAS# 554-00-7) were obtained from Alfa Aesar (Ward Hill, MA). The internal standard, zileuton [N-(1-benzobthien-2-ylethyl)-N-hydroxyurea] was obtained from Rhodia Pharma Solutions Ltd (Cramlington, Northd, UK). Formic acid was purchased from Avantor Performance Materials, Inc. (Center Valley, PA). Methanol was LC–MS grade and purchased from Honeywell Burick & Jackson (Muskegon, MI). Water was filtered and deionized with a US Filter PureLabPlus UV/UF system used throughout in all aqueous solutions. Drug-free (blank) human plasma from six different healthy donors was obtained from Innovative Research Inc. (Novi, MI, USA).

2.2. Stock solutions and standards

Stock solutions of UTL-5g, ISOX, DCA and zileuton (internal standard) were prepared in methanol at a concentration of 1 mg/mL, and stored in glass vials at -20°C . Working stock solutions were prepared fresh on each day of analysis as serial dilutions in methanol. The calibration curves were constructed by spiking analyte mix in blank plasma at the concentrations of 0.05, 0.10, 0.20, 0.50, 1.0, 2.0, 5.0, and 10.0 μM . Quality control (QC) samples were spiked in blank plasma at UTL-5g concentration of 0.05 (LLOQ), 0.15, 4 and 8 μM , at DCA concentration of 0.1, 0.3, 4 and 8 μM , and at ISOX concentration of 0.2, 0.6, 4 and 8 μM , respectively. All standards and QC samples were prepared fresh daily. For long-term and freeze-thaw stability, QC samples were prepared as a batch and stored at -80°C .

2.3. Sample preparation

Prior to preparation, frozen samples were thawed at ambient temperature. A 100 μL aliquot of plasma was added to a 1.5-mL polypropylene Eppendorf tube followed by precipitation with 300 μL of methanol containing internal standard zileuton (100 nM). The mixture was vortex-mixed for approximate 1 min, and centrifuged at 14,000 rpm for 5min at ambient temperature. The supernatant was transferred into an autosampler vial equipped with a 150- μL glass limited-volume insert, sealed with rubber/Teflon screw cap. A volume of 10 μL was injected onto the HPLC instrument using a temperature-controlled autosampling device (set at 4°C).

2.4. Chromatographic and mass-spectrometric conditions

A Waters Model 2695 separations LC system coupled with a Waters Quattro MicroTM triple quadrupole mass-spectrometry (Milford, MA, USA) was used. UTL-5g, its metabolites DCA and ISOX, and the internal standard zileuton were separated from potentially interfering material on a Nova-Pak C₁₈ column (4 μm , 3.9 mm $\times$ 150 mm; Waters Corp., Milford, MA) maintained at 30°C . The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B), pumped at a flow rate of 0.2 mL/min. A gradient program was used for the separation and identification of all tested compounds. The program was initiated with 10–95% B from 0 to 3.5 min, hold at 95% till 19 min, 95 to 10% B from 19 to 19.5 min, and 10% B from 19.5 to 24 min. The injection volume was 10 μL . The total run time was 24 min. All analytes were baseline separated.

The Waters Quattro MicroTM triple quadrupole mass-spectrometry (Milford, MA, USA) was operated in the positive electrospray ionization (ESI) mode. The instrument was controlled by the Masslynx 4.1 software. Nitrogen was used as desolvation gas. The ion source temperature was 120°C . The desolvation temperature is 350°C with a gas flow rate of 500 L/h. Argon was used as collision gas at a pressure of 0.00172 mBar. The capillary voltage was set at 3 kV. The dwell time per channel was 0.05 s for data collection. The analytes were detected in multiple reactions monitoring (MRM) mode. Optimal mass spectrometric settings (cone voltage, fragment and collision energy) for each transition were chosen and confirmed both through a series of flow injections and by comparing the peak under different conditions. The optimal settings are listed in Table 1.

2.5. Method validation

The method was validated for specificity, linearity, accuracy, precision, matrix effect and recovery, and stability according to the FDA guideline for validation of analytical methods [8].

2.5.1. Specificity—The specificity of the method was tested by visual inspection of chromatograms of processed human plasma samples from 6 different donors for the presence of endogenous or exogenous interfering peaks. The interfering peak area needed to be less than 20% than the peak area for the analytes at the LLOQ in plasma.

2.5.2. Calibration curve—Linearity was assessed at the analyte (UTL-5g, DCA or ISOX) concentration ranging from 0.05 to 10 μM . Calibration curves were built by fitting the analyte concentrations of the calibrators *versus* peak area ratios of the analyte to internal standard using the linear least squares regression analysis with different weighting scheme (*i.e.*, 1, 1/ x , and 1/ x^2). The selection of weighting scheme was guided by evaluation of goodness-of-fit criteria including correlation coefficient (R^2), % recovery of back-calculated calibrators and QCs, and residual plots.

2.5.3. Accuracy and precision—Validation runs for the calibrator standards (in duplicate) and QCs (in quintuplicate) including LLOQ, low, medium, and high were performed on three consecutive days. The accuracy was assessed as the relative percentage of the back-calculated to nominal concentration, which was equal to determined concentration/nominal concentration $\times 100\%$. The within- and between-day precisions were estimated by one-way analysis of variance (ANOVA) using the JMPTM statistical discovery software version 5 (SAS Institute, Cary, NC, USA). The between-day variance (VAR_{bet}), the within-day variance (VAR_{wit}), and the grand mean (GM) of the observed concentrations across runs were calculated from ANOVA analysis. The within-day precision (WDP) was calculated as:

$$\text{WDP} = 100 \times \left(\frac{\sqrt{\text{VAR}_{\text{wit}}}}{\text{GM}} \right)$$

The between-day precision (BDP) was defined as:

$$\text{BDP} = 100 \times \left(\frac{\sqrt{((\text{VAR}_{\text{bet}} - \text{VAR}_{\text{wit}})/n)}}{\text{GM}} \right)$$

where n represents the number of replicate observations within each day [8].

2.5.4. Matrix effect and recovery—To assess the possibility of ionization suppression or enhancement for the determination of UTL-5g, ISOX and DCA, matrix effect and recovery were assessed in 6 different donors of human plasma as described previously with modifications [9,10]. Three sets of QC samples (including low, medium and high QCs) were prepared in blank human plasma (Set 3). An aliquot of 100 μl of each Set 3 plasma samples

was precipitated by adding 300 μ L of methanol, followed by vortex-mixing and centrifugation (at 14,000 rpm for 5 min), and 10 μ L of supernatant was injected into the HPLC. The same amounts of the analytes as in Set 3 samples were spiked into 100 μ L of the mobile phase (Set 1) and 100 μ L of blank plasma extracts (*i.e.*, post-precipitation supernatant solution of blank plasma) (Set 2), respectively. An aliquot of 10 μ L of each Set 1 and Set 2 samples was directly injected into the HPLC. The matrix effect is expressed as the ratio of the mean peak area of an analyte spiked post-precipitation (Set 2) to that from neat solution (Set 1). The recovery is calculated as the ratio of the mean peak area of an analyte spiked prior to precipitation (Set 3) to that from post-precipitation solution (Set 2).

2.5.5. Stability—The short-term (bench-top) stability of each analyte (UTL-5g, DCA, or ISOX) in methanol (working solution) at the concentration of 1 and 100 μ M as well as in plasma at the concentrations of LQC and HQC was tested at ambient temperature (25 °C) for up to 6 h. The autosampler stability of all analytes (UTL-5g, DCA, and ISOX) in the precipitation solution (methanol, 1:3, v/v) was examined at 4 °C for 16 h after the LQC and HQC plasma samples were processed. The freeze–thaw stability of all analytes in plasma was assessed at the concentrations of LQC and HQC through three freeze–thawing cycles. The long-term stability of the analyte at LQC and HQC was investigated up to 3 months. All QCs were run in triplicate.

3. Results and discussion

3.1. Mass spectrometric detection and chromatographic separation

The mass spectrum of UTL-5g, DCA and ISOX showed protonated molecules ($[MH^+]$) at m/z 271.17, 161.92, and 128.05, respectively. The collision energy fragmented the analytes into several fragments. The major fragments observed at m/z 109.96, 125.95, and 109.96 were selected for subsequent monitoring in the third quadrupole for UTL-5g, DCA and ISOX, respectively (Fig. 1). The internal standard, zileuton, had protonated molecules ($[MH^+]$) at m/z 237.08 and produced a major fragment at m/z 161.03. The LC–MS/MS parameters for quantitation of all analytes were listed in Table 1.

The extracted ion chromatogram of UTL-5g, DCA, and ISOX is shown in Fig. 2. An adequate chromatographic separation of three analytes was achieved. The mean ($\pm$ standard deviation) retention times for UTL-5g, DCA, ISOX and zileuton under the optimal chromatographic conditions were at 16.86 ± 0.02 , 14.60 ± 0.02 , 11.85 ± 0.01 , and 13.53 ± 0.02 min, respectively (Fig. 2). In spite of the high specificity of MS/MS, it has been well recognized that co-eluted analytes (ions) may cause significant matrix effect (ion suppression) in some cases. To avoid potential ion suppression and interference, one goal of our assay development was to achieve adequate chromatographic separation of three analytes. After testing a variety of HPLC columns and mobile phase conditions, we found that the present chromatographic condition with a running time of 24 min produced both high-resolution chromatographic separation and high-sensitivity electrospray ionization signal though the analysis speed was compromised. Representative chromatograms of both blank and analytes spiked in human plasma samples at LLOQ are shown in Fig. 3. The selectivity for the analysis was shown by symmetrical resolution of the peaks, with no

significant chromatographic interference at the expected retention times of the analytes from six different donors of blank human plasma (Fig. 3).

3.2. Calibration curves

The calibration curves were established over the concentration range of 0.05–10 μM for UTL-5g, 0.1–10 μM for DCA, and 0.2–10 μM for ISOX. The relationship between peak area ratios of the analyte to internal standard *versus* the analyte concentrations was best fitted by a linear equation, expressed as $y = a \cdot x + b$, where y is peak area ratio, x is the analyte concentration, a and b are fitted parameters. A weighting function of $1/x^2$ produced the best goodness-of-fit. For all analyte curves, a correlation coefficient (R^2) of >0.99 was obtained in all analytical runs, and the distribution of residuals was random and centered on zero (data not shown). As shown in Table 2, the average accuracy of the calibrator standards in terms of % recovery of the back-calculated relative to nominal concentration ranged from 98.3% to 102.3% ($n = 6$) for UTL-5g, from 97.4% to 102.1% ($n = 6$) for DCA, and from 94.8 to 103.5 ($n = 6$) for ISOX, respectively; the within- and between-day precisions were less than 12.7%, 11.7, and 9.6% for all calibrator standards of UTL-5g, DCA, and ISOX, respectively.

3.3. Accuracy and precision

The LLOQ for UTL-5g, DCA, and ISOX was established at 0.05, 0.1, and 0.2 μM , at which the mean signal-to-noise ratio was 36 ($n = 15$), 12 ($n = 15$), and 11 ($n = 15$), respectively. For the QCs of UTL-5g at the concentrations of 0.05 (LLOQ), 0.15, 4, and 8 μM , the average accuracy ranged from 87.3% to 97.2%, and within- and between-day precisions were all less than 11.9% (Table 3). For the QCs of DCA at the concentrations of 0.1 (LLOQ), 0.3, 4, and 8 μM , the average accuracy was determined from 89.5% to 102.6%, and within- and between-day precisions were within 11.5%. For the QCs of ISOX at the concentrations of 0.2 (LLOQ), 0.6, 4, and 8 μM , the average accuracy ranged from 93.3% to 104.8%, and within- and between-day precisions were less than 11.7% (Table 3).

Usually, the concentrations of metabolites in biology samples are lower than their parent compound, thus higher sensitivities of the metabolites may be helpful. In this case, the LLOQs of Isox and DCA are 0.2 and 0.1 μM respectively, higher than that of UTL-5g, 0.05 μM . This is partially due to the lower molar extinction coefficient of either Isox or DCA as compared to the parent drug, UTL-5g, which comprises the chromophores from both ISOX and DCA. At the present time, it is not clear whether the sensitivity of ISOX or DCA is sufficient for clinical samples. In case it becomes an issue in the future, modifications of the current method may be needed.

3.4. Matrix effect and recovery

Matrix effects for the analytes and internal standard were evaluated in 6 different donors of human plasma to assess the possibility of ionization suppression or enhancement. A slight ion suppression was observed for UTL-5g (mean matrix factor, 0.89) and DCA (mean matrix factor, 0.77), and a moderate ion suppression was observed for ISOX (mean matrix factor, 0.41) (Table 4). The interindividual variability in the matrix effect for UTL-5g, DCA, and ISOX among 6 different sources of plasma, as measured by the coefficient of variation (CV %), were ranging from 3.5% to 16.2% across different QC concentrations (Table 4).

This degree of variations is within the generally acceptable criteria for bioanalytical assays. The matrix effect for individual analyte (UTL-5g, DCA, or ISOX) was consistent across different QC concentrations (with CV% < 10%).

The extraction recovery of all three analytes (at the low, medium, and high QC concentrations) and the internal standard from 6 different donors of plasma ranged from 98% to 110%, with the interindividual variability within 15% (Table 4). These data suggested that all the analytes and internal standard were completely and consistently extracted by protein precipitation.

3.5. Stability

The short term stability of UTL-5g, DCA and ISOX was demonstrated in Table 5. At ambient temperature (~25 °C), all analytes were stable for at least 6 h in stock solution at the concentrations of 1 and 100 µM. In plasma samples, ISOX and DCA were stable for up to 6 h at the concentrations of LQC and HQC. However, UTL-5g was stable in plasma for 2 h at HQC, but not stable at the LQC within 1 h. Therefore, to prevent the degradation of UTL-5g, it is important to keep all plasma samples and working solutions on ice during sample preparation. In the precipitation solution, UTL-5g, DCA and ISOX were stable in the autosampler (4°C) for 8 h so that the sample run was allowed to be performed continuously within 8 h (Table 3). Freeze-thaw stability, which was assessed at the analyte plasma concentrations of LQC and HQC, showed no significant degradation of DCA or ISOX through three full cycles of freeze-thaws, but some degradation (>15%) of UTL-5g at the third cycle was observed. The free-thaw stability tests suggested that UTL-5g plasma samples were stable in two cycles. Long-term stability test suggested that UTL-5g and ISOX was stable in human plasma at -80 °C for at least 3 months, but DCA was not stable, with 30–50% being degraded within 3-month storage at -80 °C.

4. Conclusion

In summary, a sensitive and reliable LC–MS/MS method was developed and validated for the determination of UTL-5g and its metabolites (DCA and ISOX) simultaneously in pooled human plasma. The LLOQ for UTL-5g, DCA and ISOX was determined at 0.05, 0.1, and 0.2 µM in plasma, respectively. Calibration curves were established in the range of 0.05–10 µM for UTL-5g, 0.1–10 µM for DCA, and 0.2–10 µM for ISOX. The intra- and inter-day precision and accuracy were within the generally accepted criteria for bioanalytical method.

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Fig. 1.

Product ion mass spectra of (A) UTL-5g with monitoring at m/z 271.17 > 109.96; (B) ISOX with monitoring at m/z 128.05 > 109.96; (C) DCA with monitoring at m/z 161.92 > 125.95 and (D) the internal standard zileuton at m/z 237.08 > 161.03.

Fig. 2.

A total ion chromatogram of a standard mixture of UTL-5g (at m/z of 271.17 > 109.96), ISOX (at m/z of 128.05 > 109.96), DCA (at m/z of 161.92 > 125.95) and the internal standard zileuton (at m/z of 237.08 > 161.03) at the concentration of 1 μ M.

Chromatographic separation was performed on a Nova-Pak C18 column (4 μ m, 3.9 mm $\times$ 150 mm) at 30°C, running with mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) at the flow rate of 0.2 mL/min. Mobile phase gradient B%(min) was programmed as 10 (0) $\rightarrow$ 95(3.5) $\rightarrow$ 95 (19) $\rightarrow$ 10(19.5) $\rightarrow$ 10(24). The retention times were 16.86 ± 0.02 for UTL-5g, 11.85 ± 0.01 for ISOX and 14.60 ± 0.02 for DCA, and 13.53 ± 0.02 for internal standard zileuton.

Fig. 3.

Chromatograms of blank plasma (A, C and E) and plasma spiked with (B) UTL-5g (LLOQ, 0.05 μ M), (D) ISOX (LLOQ, 0.20 μ M) and (F) DCA (LLOQ, 0.1 μ M). Chromatographic separation was performed on a Nova-Pak C18 column (4 μ m, 3.9 mm $\times$ 150 mm) at 30 $^{\circ}$ C, running with mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) at the flow rate of 0.2 ml/min. Mobile phase gradient B%(min) was programmed as 10 (0) $\rightarrow$ 95 (3.5) $\rightarrow$ 95 (19) $\rightarrow$ 10 (19.5) $\rightarrow$ 10 (24). The retention times were 16.86 ± 0.02 min for UTL-5g, 11.85 ± 0.01 min for ISOX and 14.60 ± 0.02 min for DCA. The transitions were monitored at m/z of 271.17 $>$ 109.96 for UTL-5g, 128.05 $>$ 109.96 for ISOX, 161.92 $>$ 125.95 for DCA.

Table 1

LC-MS/MS parameters for quantitation of UTL-5g and its metabolites (DCA and ISOX).

	UTL-5g	DCA	ISOX	Zileuton (IS)
MS mode	Positive	Positive	Positive	Positive
<i>m/z</i> transition	271.17 > 109.96	161.92 > 109.96	128.05 > 109.96	237.08 > 161.03
Capillary voltage (Kv)	3	3	3	3
Cone voltage (V)	20	35	15	16
Collision energy (V)	16	18	10	12
Desolvation temperature (°C)	350	350	350	350
Source temperature (°C)	120	120	120	120
Retention time (min)	16.86	14.63	11.85	13.53
Mobile phase gradient program: %B (min)	10 (0) → 95 (3.5) → 95 (19) → 10 (19.5) → 10 (24)			

Table 2

Accuracy, within- and between-day precisions of calibrator standards^a of the calibration curves of UTL-5g and its metabolites in plasma.

Compound	Nominal concentration (µM)	Determined concentration (µM)	Average accuracy (%)	Within-day (%)	Between-day (%)
UTL-5g	0.05 (LLOQ)	0.051 ± 0.002	102.0	2.8	3.4
	0.1	0.10 ± 0.01	98.3	12.5	3.2
	0.2	0.20 ± 0.01	98.5	4.6	2.1
	0.5	0.50 ± 0.04	100.9	8.9	<i>b</i>
	1.0	1.00 ± 0.09	99.9	10.9	<i>b</i>
	2.0	2.04 ± 0.20	102.2	12.0	<i>b</i>
	5.0	4.93 ± 0.48	98.7	12.7	<i>b</i>
DCA	10.0	10.23 ± 1.01	102.3	12.5	<i>b</i>
	0.1 (LLOQ)	0.098 ± 0.006	97.7	7.9	<i>b</i>
	0.2	0.20 ± 0.02	102.1	11.7	<i>b</i>
	0.5	0.50 ± 0.04	100.0	8.7	<i>b</i>
	1.0	0.98 ± 0.06	97.9	8.0	<i>b</i>
	2.0	2.03 ± 0.15	101.7	3.5	7.2
	5.0	4.87 ± 0.32	97.4	8.2	<i>b</i>
ISOX	10.0	10.20 ± 0.68	102.0	8.6	<i>b</i>
	0.2 (LLOQ)	0.19 ± 0.02	94.8	5.9	9.6
	0.5	0.49 ± 0.04	97.1	4.3	6.0
	1.0	1.01 ± 0.02	100.6	2.1	0.9
	2.0	1.99 ± 0.15	99.5	7.5	<i>b</i>
	5.0	4.97 ± 0.17	99.4	4.2	<i>b</i>
	10.0	10.35 ± 0.41	103.5	5.0	<i>b</i>

^aEach calibrator standard was evaluated in duplicate on three different days.

No additional variation was observed as a result of performing assay in different days.

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Table 3

Accuracy, within- and between-day precisions for the QC samples^a of UTL-5g and its metabolites in human plasma.

Compound	Nominal concentration (µM)	Determined concentration (µM)	Average accuracy (%)	Within-day (%)	Between-day (%)
UTL-5g	0.05 (LLOQ)	0.045 ± 0.01	90.3	8.3	7.4
	0.15	0.131 ± 0.01	87.3	5.5	^b —
	4.0	3.73 ± 0.43	93.2	10.0	6.9
	8.0	7.77 ± 0.99	97.2	6.6	11.9
DCA	0.1 (LLOQ)	0.099 ± 0.01	99.4	10.0	3.1
	0.3	0.27 ± 0.03	89.5	11.5	3.6
	4.0	4.09 ± 0.34	102.2	5.1	7.7
	8.0	8.21 ± 0.91	102.6	6.9	10.2
ISOX	0.2 (LLOQ)	0.19 ± 0.02	96.7	11.7	1.6
	0.6	0.56 ± 0.05	93.3	5.3	8.9
	4.0	4.10 ± 0.34	102.6	3.4	8.8
	8.0	8.38 ± 0.78	104.8	5.4	8.9

^a Each QC was performed in quintuplicate on three different days.

Table 4

Matrix effect and recovery of UTL-5g, its metabolites and zileuton (IS) from 6 different donors of human plasma.

Analyte	Nominal concentration (μM) ^a	Mean peak area		Matrix effect (%) ^e	Recovery (%) ^f
		Set 1 ^b	Set 2 ^c		
UTL-5g	0.15	753	683	655	97.8 (14.5)
	4.0	23078	20540	19956	97.5 (13.5)
	8.0	38275	31859	32606	102.6 (5.5)
DCA	0.3	73.1	51.6	50.9	100.3 (11.1)
	4.0	1144.1	821.4	900.1	109.7 (4.8)
	8.0	2180.3	1798.7	1880.8	104.7 (2.6)
ISOX	0.6	3055.2	1241.4	1313.2	106.2 (4.9)
	4.0	20377.2	8273.8	8735.4	105.6 (2.9)
	8.0	40355.5	17099.4	18021.4	105.4 (1.7)
Zileuton	0.1	3856	2619	2554	98.0 (2.6)

^aThe nominal concentrations of the analyte spiked in plasma before precipitation (Set 3). The same amounts of the analyte as in Set 3 were spiked in the mobile phase and in plasma extract for Set 1 and Set 2.

^bThe mean peak area of an analyte that was spiked in the mobile phase from duplicate measurements.

^cThe mean peak area of an analyte that was spiked post-precipitation in plasma extracts from 6 different donors, and each source of plasma in duplicate measurements.

^dThe mean peak area of an analyte that was spiked before precipitation in plasma from 6 different sources (donors), each source of plasma in duplicate measurements.

^eMatrix effect is expressed as the ratio of the mean peak area of an analyte spiked postprecipitation (Set 2) to the mean peak area of the same amount of analyte spiked in the mobile phase (Set 1). Data are shown as the mean (%CV) from six different sources of plasma.

^fRecovery is calculated as the ratio of the mean peak area of an analyte spiked before precipitation (Set3) to the mean peak area of the same amount of the analyte spiked postextraction (Set 2). Data are shown as the mean (%CV) from six different source of plasma.

Table 5

Assessment of stability of UTL-5g and its metabolites ISOX and DCA.

Bench-top stability (in stock solution) (25 °C) (%) ^d	UTL-5g (µM) ^c			ISOX (µM) ^c			DCA (µM) ^c		
	1	100	1	100	1	100	1	100	1
1.0 h	96.5	94.2	99.8	101.6	90.2	94.4			
2.0 h	93.8	94.3	96.4	95.7	89.7	89.2			
3.0 h	91.1	94.7	96.0	95.7	95.1	91.0			
4.0 h	92.8	93.7	94.6	97.5	87.0	98.0			
6.0 h	91.5	91.8	96.6	96.3	90.0	95.6			
Bench-top stability (in plasma) (25 °C) (%) ^d	UTL-5g (µM) ^c			ISOX (µM) ^c			DCA (µM) ^c		
	0.15	8	0.6	8	0.3	8	0.3	8	8
1.0 h	72.8	95.9	96.3	90.7	91.8	92.9			
2.0 h	76.7 ^d	97.4	94.1	94.5	88.3	93.7			
3.0 h	67.5	70.9	93.2	96.2	107.6	89.0			
4.0 h	53.6	66.9	95.8	95.2	84.3	88.4			
6.0 h	50.3	60.4	100.4	96.0	93.6	86.6			
Auto-sampler stability (in the precipitation solution) (4 °C) (%) ^d	UTL-5g (µM) ^c			ISOX (µM) ^c			DCA (µM) ^c		
	0.15	8	0.6	8	0.3	8	0.3	8	8
1.0 h	99.1	94.3	86.7	98.6	110.9	76.6			
2.0 h	92.0	89.7	91.3	98.6	93.4	76.1			
4.0 h	91.8	86.9	99.0	99.6	111.4	93.8			
6.0 h	93.9	85.1	94.1	101.6	85.5	91.0			
8.0 h	87.2	80.6	107.7	101.9	131.5	94.1			
12.0 h	76.3	70.4	112.5	111.6	145.9	106.5			
16.0 h	76.9	69.5	124.2	118.9	164.5	118.7			
Freeze-thaw stability (in plasma) (-80 °C) (%) ^b	UTL-5g (µM) ^c			ISOX (µM) ^c			DCA (µM) ^c		
	0.15	8	0.6	8	0.3	8	0.3	8	8
Cycle 1	82.2	84.5	93.4	91.7	94.2	98.9			
Cycle 2	91.0	85.6	97.0	88.6	115.8	93.5			
Cycle 3	82.6	76.2	86.1	86.0	99.6	90.5			

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Long-term stability	UTL-5g (µM) ^c		ISOX (µM) ^c		DCA (µM) ^c	
	0.15	8	0.6	8	0.3	8
3.0 month	102.2	108.6	86.3	88.4	53.8	72.7

^a Stability data is expressed as the mean percentage of the peak area determined at certain time relative to that at time zero.

^b Stability data is expressed as the mean percentage of the analyte concentration determined at certain time point relative to the nominal concentration (%).

^c Each concentration at each time point was assessed in triplicate.