ELECTRONIC SUPPLEMENTARY MATERIAL

Rapid quantification of ruthenium(II) polypyridyl anti-cancer drugs using selective ligand dissociation LC-MS/MS method

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Table S1 LC-MS/MS conditions used for the detection of Ru1, Ru2, Ru3, and IS.

	Ru1	Ru2	Ru3	IS
Mobile Phase		Isocratic		
		5% A: H_2O (0.1% Formic acid)		
		95% B: MeOH (0.1% Formic acid)		
		Flow rate (0.5 mL min^{-1})		
		Column oven set at 40 °C		
Precursor ion (m/z)	373.2	387.2	297.1	305.2
Product ions (m/z)	157.1	308.7	181.1	178.2
	294.4	361.2	218.7	263.3
Collision energy (V)	20	27	27	33
Retention time (min)	0.45	0.40	0.38	0.64
Mass mode	H-ESI positive mode	H-ESI positive mode	H-ESI positive mode	H-ESI positive mode
Scan type	SRM[a]	SRM	SRM	SRM

[a]SRM, selected reaction monitoring

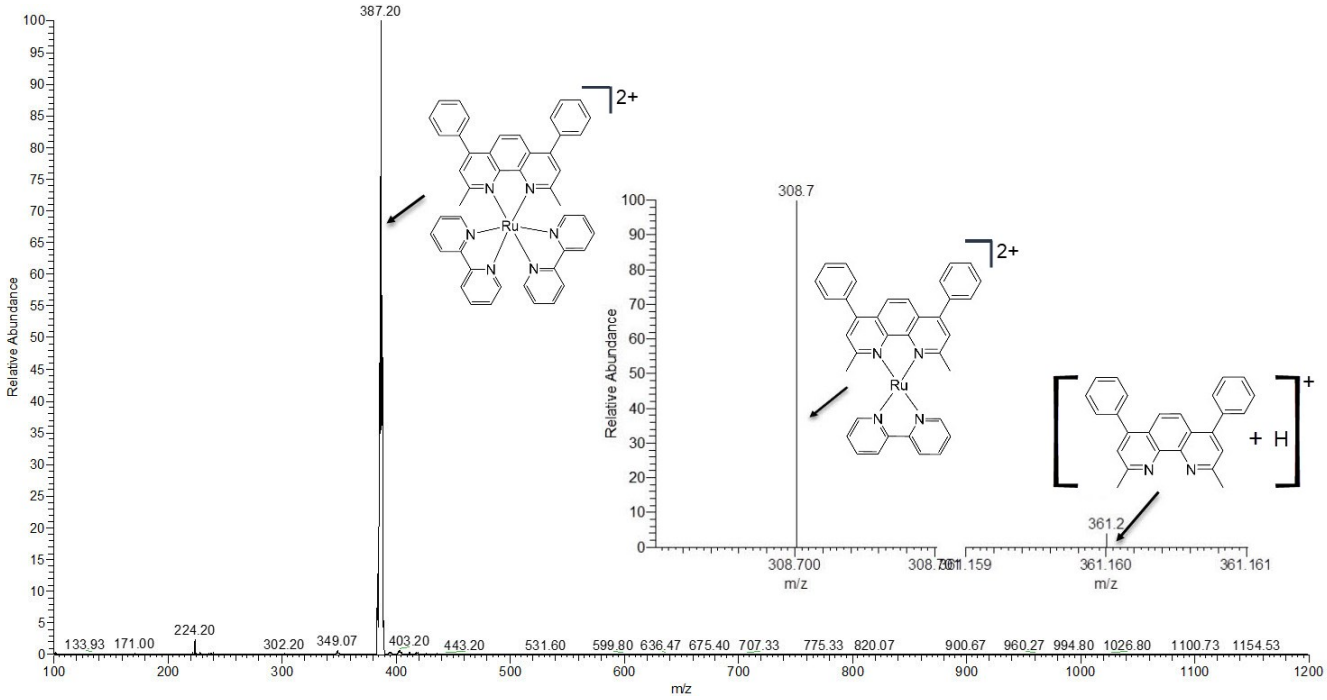

Fig. S1. LC-MS/MS spectra of Ru2. Acetonitrile was spiked with Ru2 at the lowest limit of quantification (LLOQ). Samples were then injected (1 µL) and analyzed by LC-MS/MS using the conditions listed in Table 1. Precursor ion: Ru2: 387.2; Transitions to product ions (inset): 387.2 → 308.7, 387.2 → 361.2.

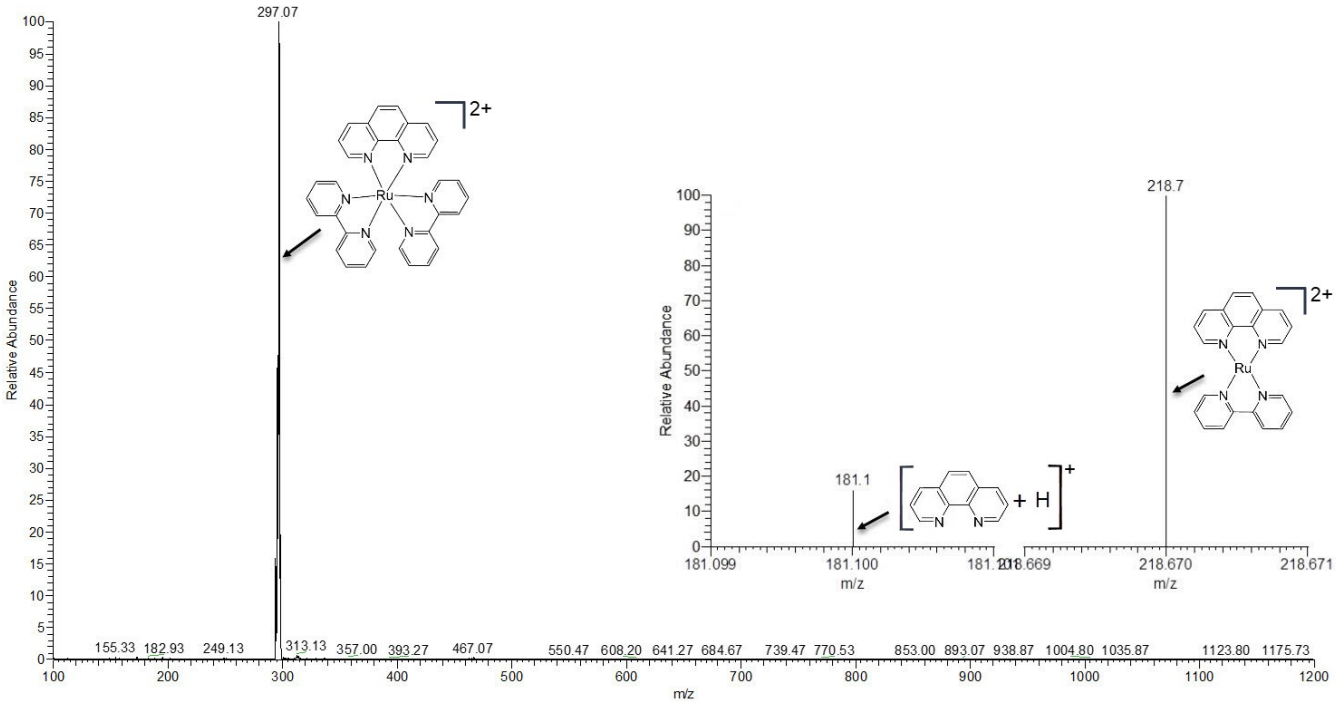

Fig. S2. LC-MS/MS spectra of Ru3. Acetonitrile was spiked with Ru3 at the lowest limit of quantification (LLOQ). Samples were then injected (1 µL) and analyzed by LC-MS/MS using the conditions listed in Table 1. Precursor ion: Ru3: 297.1; Transitions to product ions (inset): 297.1 → 181.1, 297.1 → 218.7.

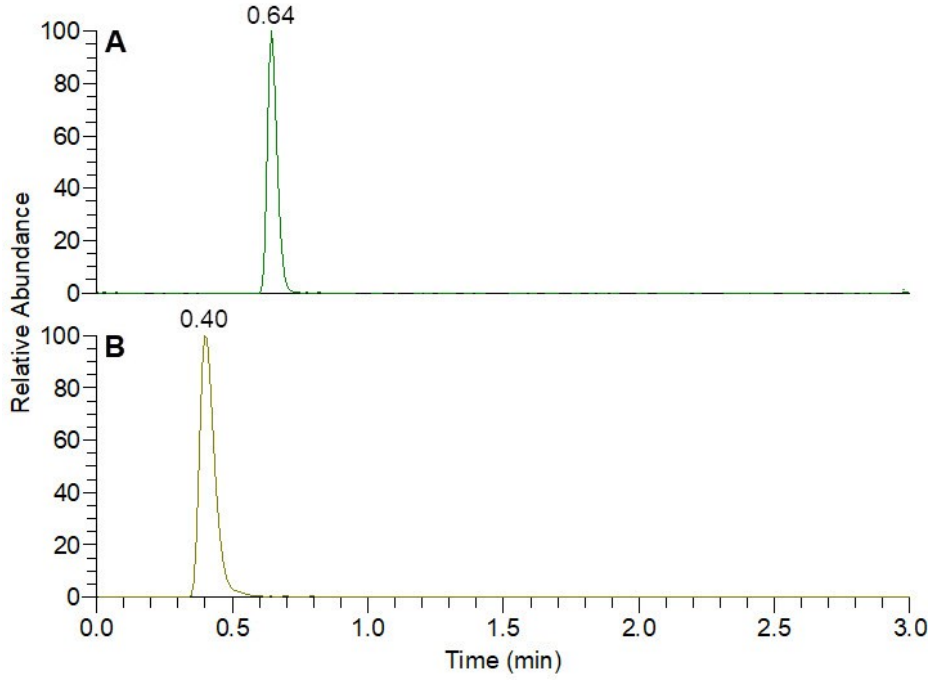

Fig. S3. Representative chromatograms of Ru2 and IS. Acetonitrile was spiked with 0.06 µM Ru2 and IS at 15 ng mL^{-1}. The resulting solution was then injected (1 µL) and analyzed by LC-MS/MS. A, IS: RT = 0.64 min; B, Ru2: RT = 0.40 min.

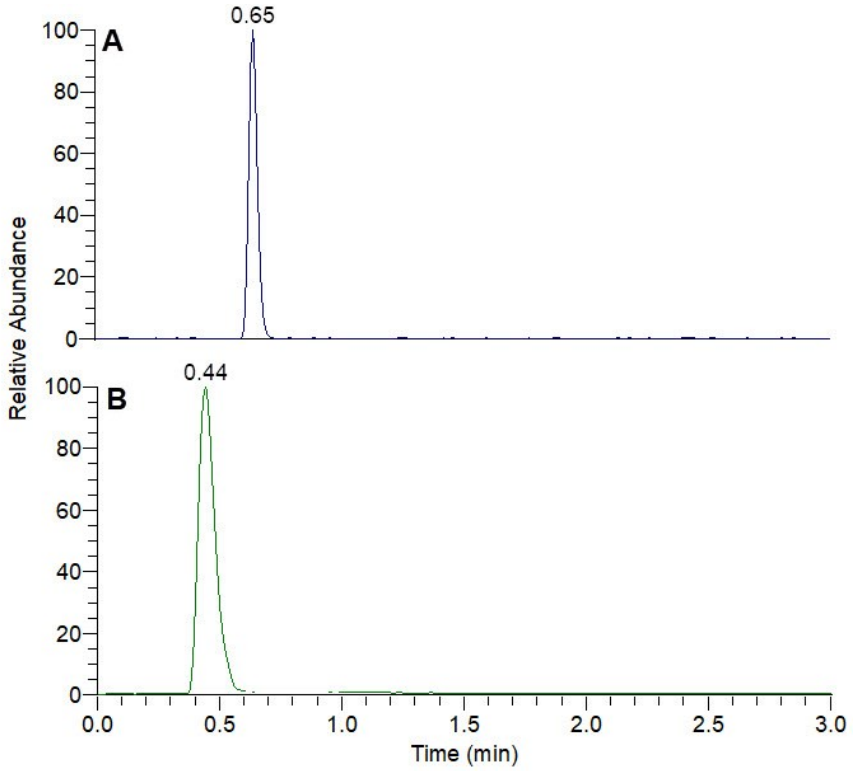

Fig. S4. Representative chromatograms of Ru3 and IS. Acetonitrile was spiked with 0.06 µM Ru3 and IS at 15 ng mL^{-1}. The resulting solution was then injected (1 µL) and analyzed by LC-MS/MS. A, IS: RT = 0.65 min; B, Ru3: RT = 0.44 min.

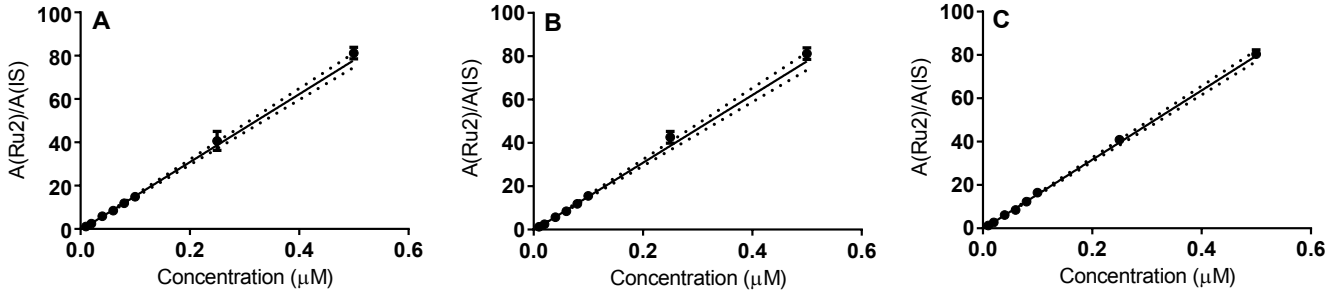

Fig. S5. Calibration curves developed as standards for the quantification of Ru2 in cells, plasma, or urine. Graphs represent the peak-area ratios of Ru2 to IS (y axis) versus the nominal concentrations of Ru2 (x axis) and curves are fitted to a line having the equation $y = ax + b$ using a weighting factor $1/x^2$, $r^2 \geq 0.99$. Concentrations were interpolated from the calibration curves and all non-zero calibrators were within ± 15% deviation from the theoretical values which complied with

validation guidelines. A, in cells, y = 156.9x - 0.5276, r^2 = 0.994; B, in plasma, y = 156.1x – 0.3955, r^2 = 0.990 and C, in urine, y = 159.9x – 0.3530, r^2 = 0.996.

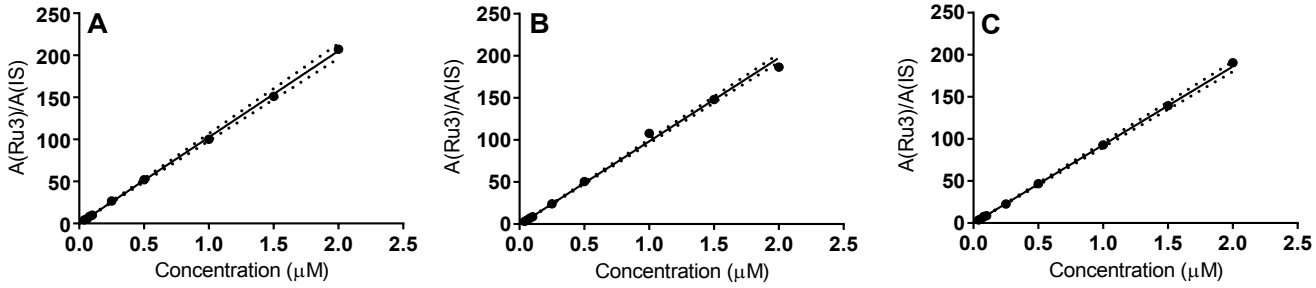

Fig. S6. Calibration curves developed as standards for the quantification of Ru3 in cells, plasma, or urine. Graphs represent the peak-area ratios of Ru3 to IS (y axis) versus the nominal concentrations of Ru3 (x axis) and curves are fitted to a line having the equation y = ax + b using a weighting factor $1/x^2$, $r^2 \geq 0.99$. Concentrations were interpolated from the calibration curves and all non-zero calibrators were within ± 15% deviation from the theoretical values which complied with validation guidelines. A, in cells, y = 102.4x - 0.0528, r^2 = 0.991; B, in plasma, y = 99.13x - 0.8471, r^2 = 0.996 and C, in urine, y = 93.1x – 0.2546, r^2 = 0.996.

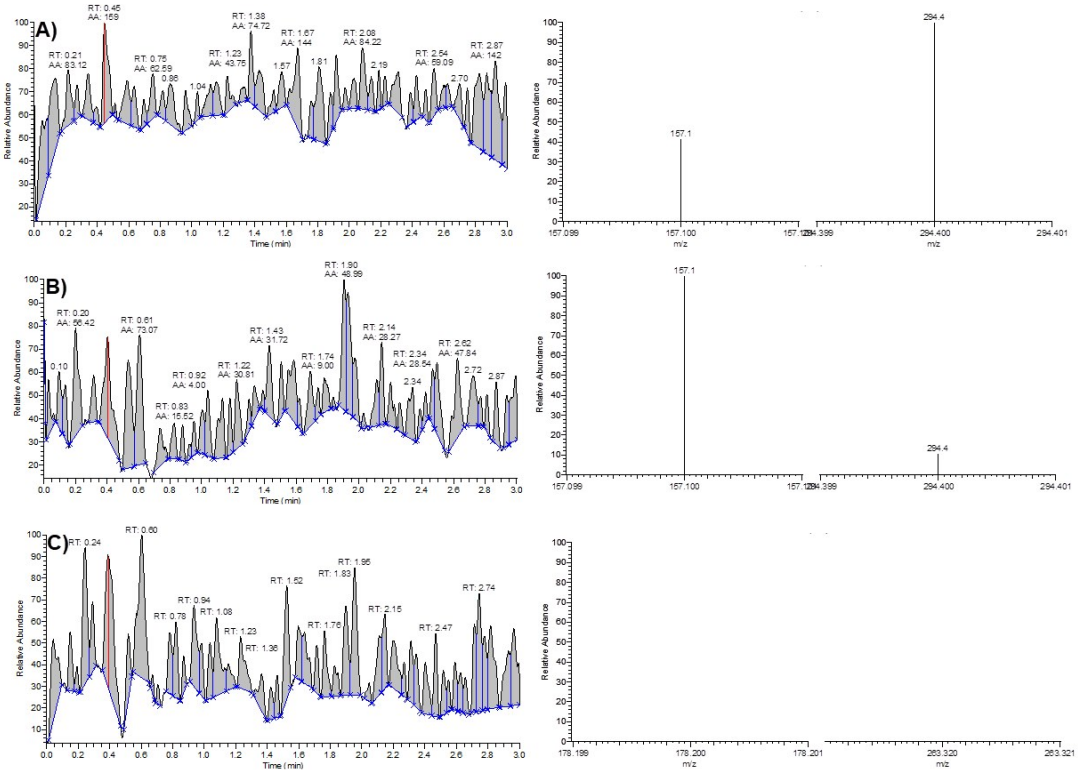

Fig. S7. HPLC chromatograms of blank samples analyzed for signal interference at the RT of Ru1. A) cells; B) plasma; C) urine. Product ion shifts expected, 373.2 → 157.1, 373.2 → 294.4.

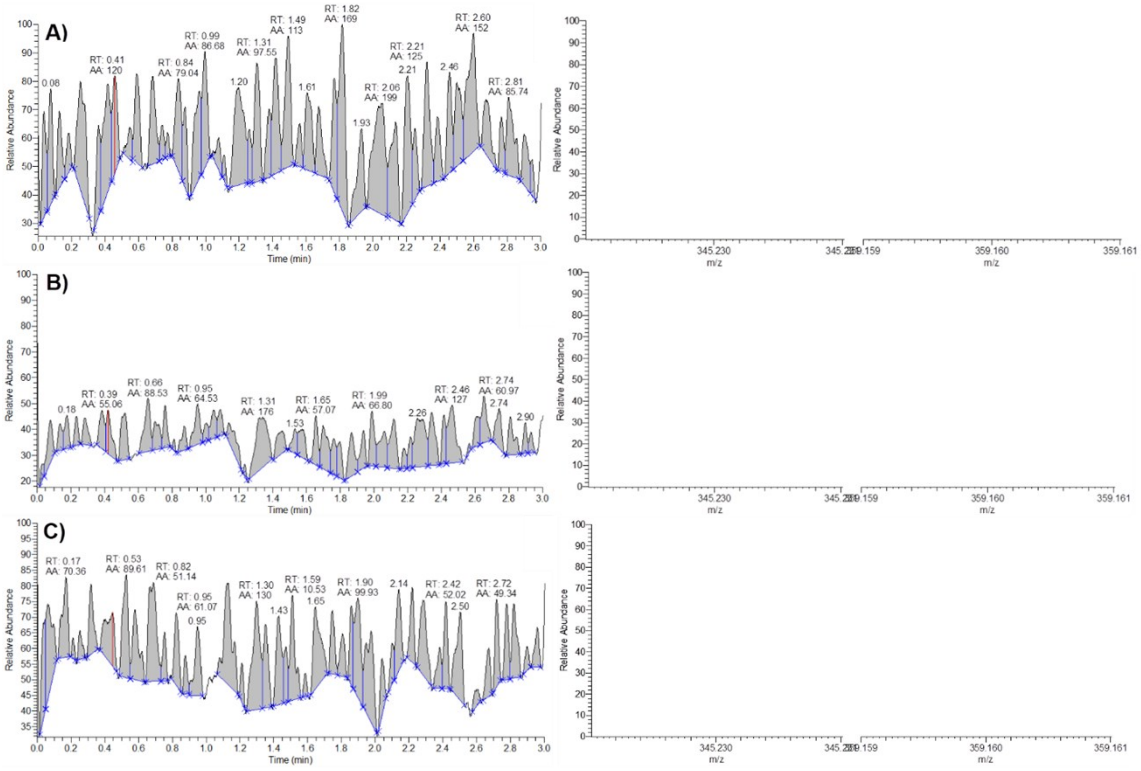

Fig. S8. HPLC chromatograms of blank samples analyzed for signal interference at the RT of Ru2. A) cells; B) plasma; C) urine. Product ion shifts expected, 387.2 → 308.7, 387.2 → 361.2.

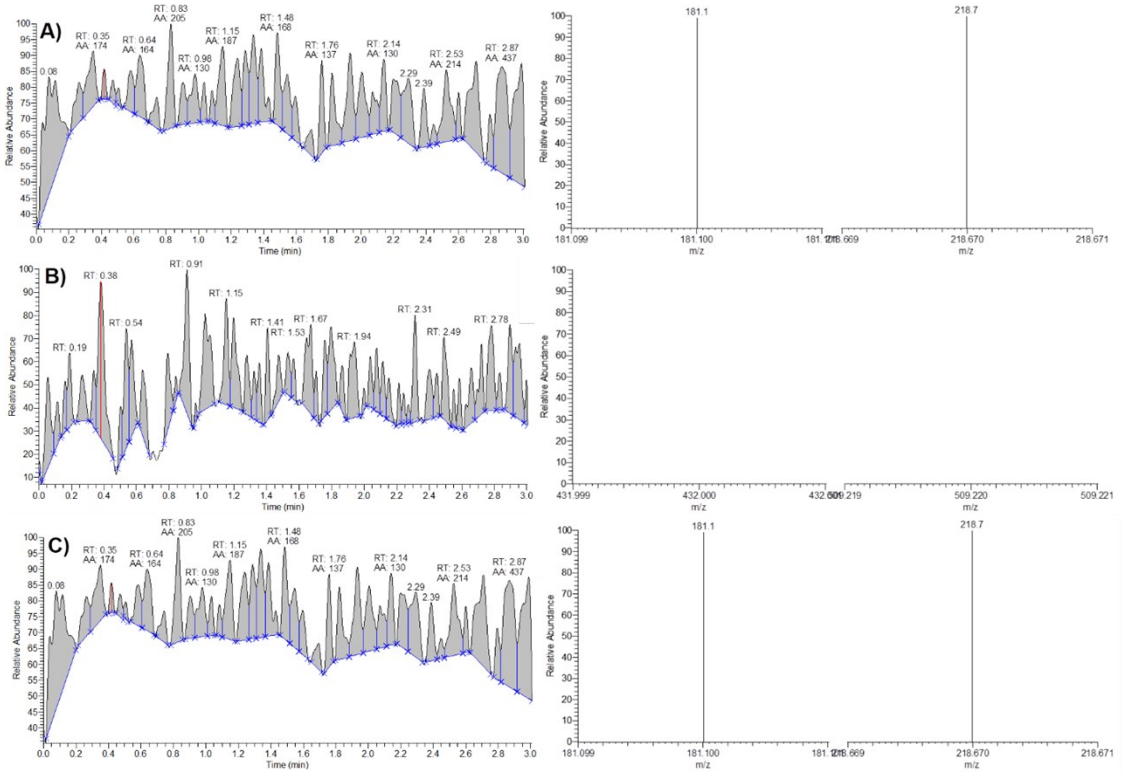

Fig. S9. HPLC chromatograms of blank samples analyzed for signal interference at the RT of Ru3. A) cells; B) plasma; C) urine. Product ion shifts expected, 297.1 → 181.1, 297.1 → 218.7.

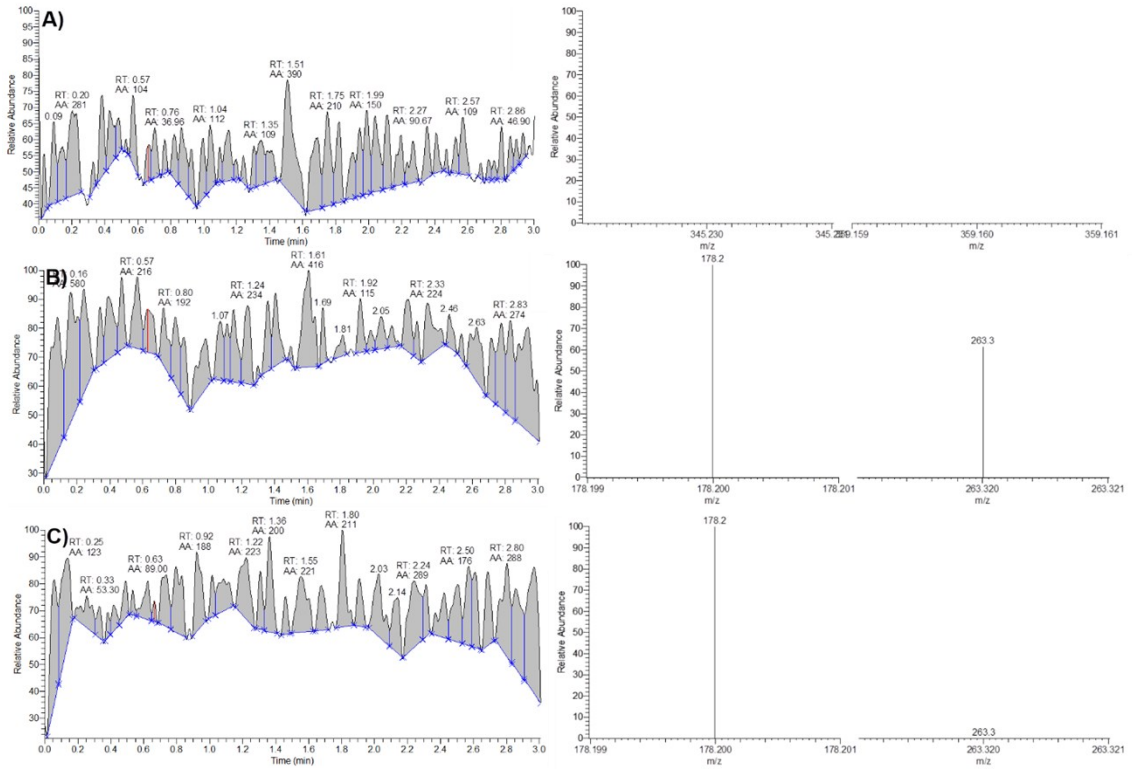

Fig. S10. HPLC chromatograms of blank samples analyzed for signal interference at the RT of the IS. A) cells; B) plasma; C) urine. Product ion shifts expected, 305.2 → 178.2, 305.2 → 263.3.

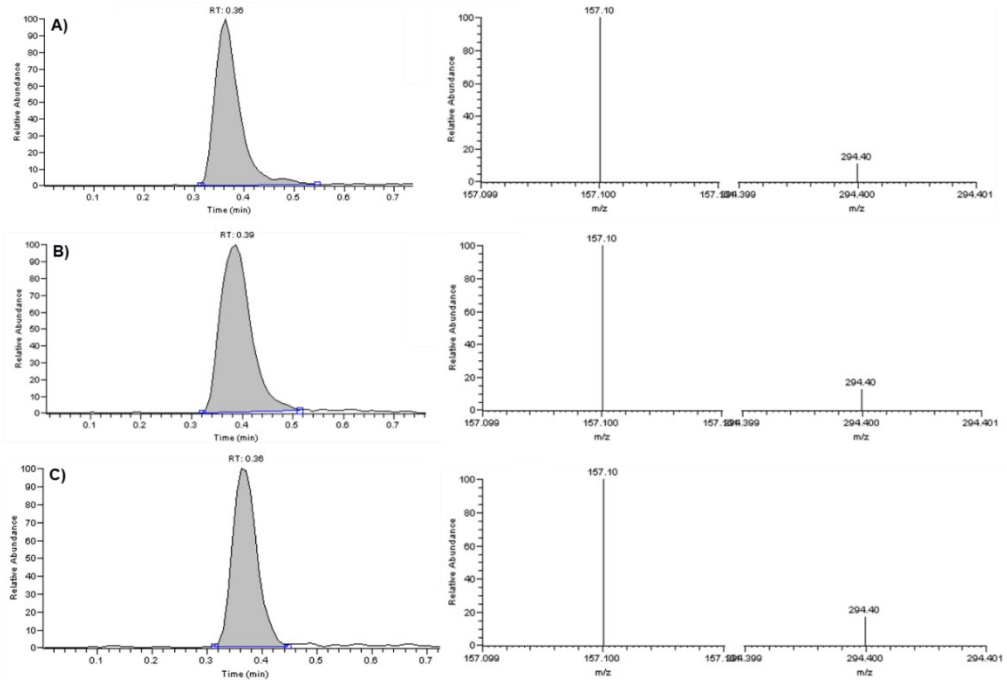

Fig. S11. HPLC chromatograms and product ion scans of Ru1 at the LLOQ. A) in cells, RT: 0.36 min; B) in plasma, RT: 0.39 min; C) in urine, RT: 0.36 min. Product ion shifts, 373.2 → 157.1, 373.2 → 294.4.

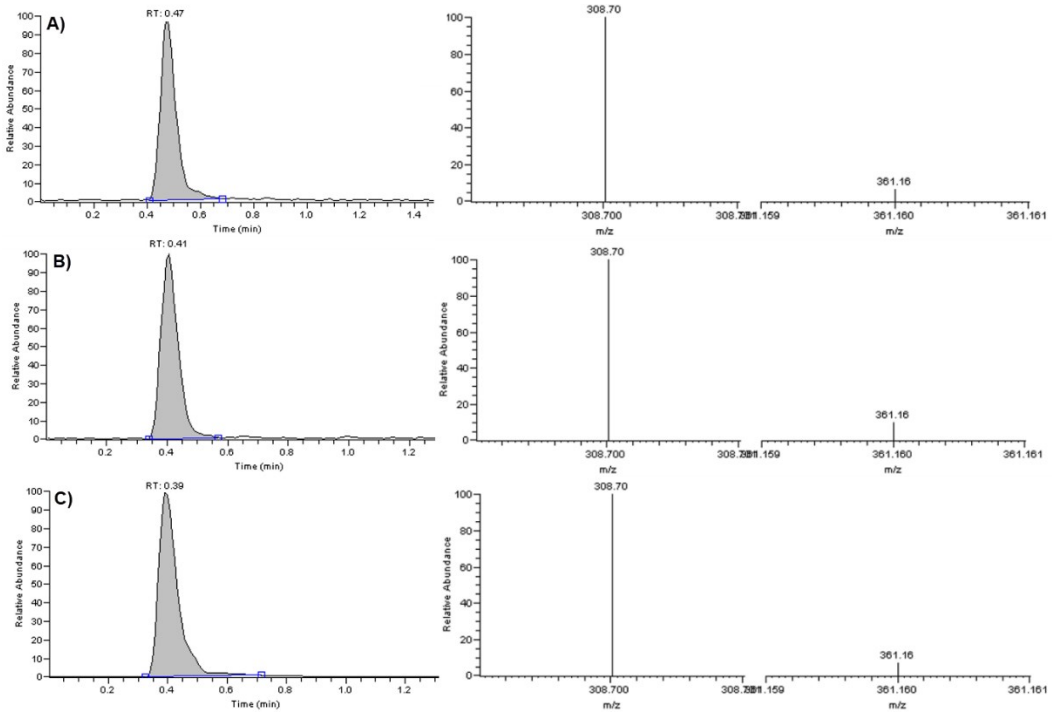

Fig. S12. HPLC chromatograms and product ion scans of Ru2 at the LLOQ. A) in cells, RT: 0.47 min; B) in plasma, RT: 0.41 min; C) in urine, RT: 0.39 min. Product ion shifts, 387.2 → 308.7, 387.2 → 361.2.

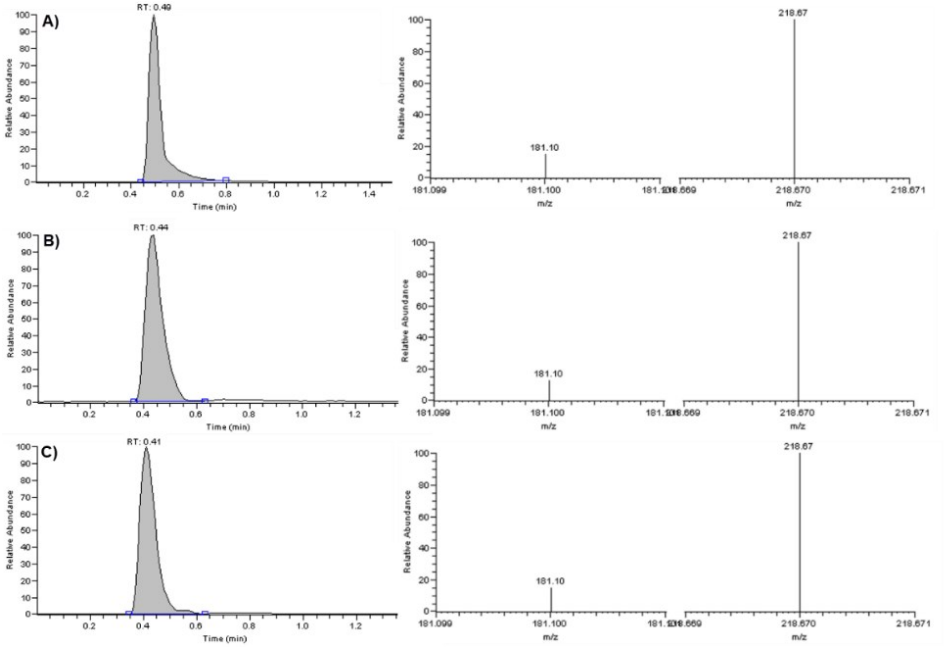

Fig. S13. HPLC chromatograms and product ion scans of Ru3 at the LLOQ. A) in cells, RT: 0.49 min; B) in plasma, RT: 0.44 min; C) in urine, RT: 0.41 min. Product ion shifts, 297.1 → 181.1, 297.1 → 218.7.

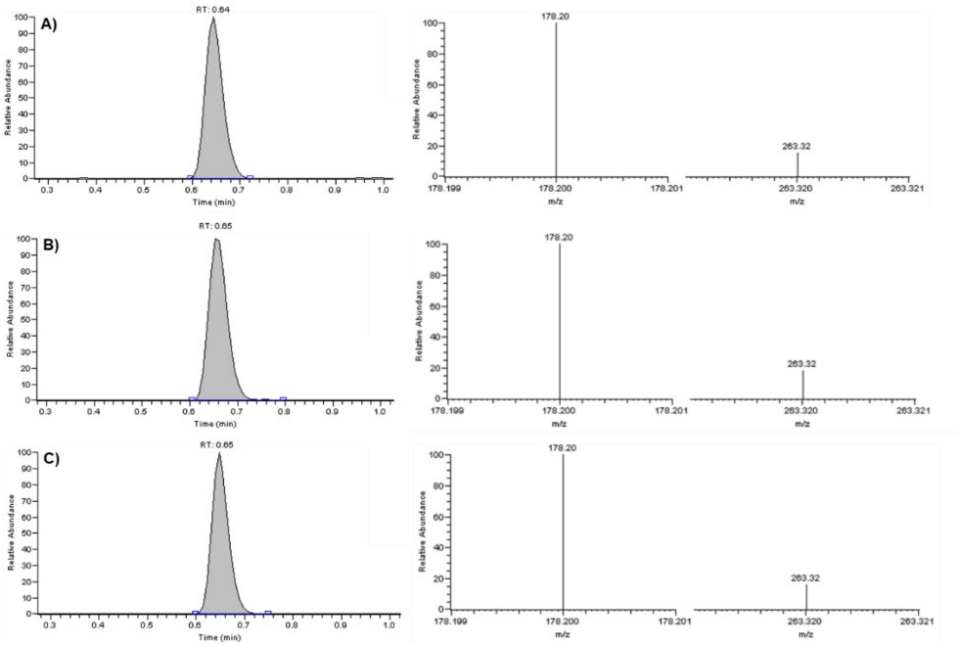

Fig. S14. HPLC chromatograms and product ion scans of the IS at 15 ng mL^{-1}. A) in cells, RT: 0.64 min; B) in plasma, RT: 0.65 min; C) in urine, RT: 0.65 min. Product ion shifts, 305.2 → 178.2, 305.2 → 263.3.

Table S2 Response of Ru1, Ru2, and Ru3 in blank samples (cells, plasma, or urine) versus samples spiked at the LLOQ. Six samples from individual sources were used at each condition and values are the mean area measured by LC-MS/MS ± SD.

Analyte	Cells			Plasma			Urine		
	Blank (Area ± SD)	Sample at LLOQ (Area ± SD)	% response[a]	Blank (Area ± SD)	Sample at LLOQ (Area ± SD)	% response[a]	Blank (Area ± SD)	Sample at LLOQ (Area ± SD)	% response[a]
Ru1	457 ± 142	23541 ± 1082	1.94	339 ± 0.00	25462 ± 2215	1.33	421 ± 96.5	25179 ± 3373	1.67
Ru2	801 ± 27.9	51497 ± 88.06	1.56	0.00 ± 0.00	59370 ± 3736	0.00	918 ± 102	53432 ± 2000	1.72
Ru3	0.00 ± 0.00	151012 ± 11824	0.00	1111 ± 304	151761 ± 2943	0.73	241 ± 85.7	151761 ± 2943	0.16

LLOQ for Ru1 and Ru2, 0.01 µM; LLOQ for Ru3, 0.04 µM.

[a] $\%\ reponse = \frac{Area\ blank}{Area\ sample\ at\ LLOQ} \times 100$

Table S3 Response of IS in blank samples (cells, plasma, or urine) versus QC samples or zero calibrators. Six samples from individual sources were used at each condition and values are the mean area measured by LC-MS/MS ± SD.

Matrix	Blank (Area ± SD)	QC samples (Area ± SD)	% response[a]	Zero calibrator (Area ± SD)	% response[a]
Cells	151 ± 23.2	45840 ± 6394	0.33	52376 ± 175	0.29
Plasma	136 ± 35.8	36850 ± 7956	0.37	32954 ± 9480	0.41
Urine	68.2 ± 25.5	48644 ± 677	0.14	38832 ± 1052	0.18

[a] $\%\ reponse = \frac{Area\ blank}{Area\ sample\ at\ LLOQ} \times 100$

Table S4 Matrix effect on the response of Ru1, Ru2, Ru3, and IS. Values represent the mean area (±SD) measured in neat standards (acetonitrile spiked with the analytes at the LQC and HQC levels or IS at 15 ng mL^{-1}) and their equivalents in samples of cells, plasma, or urine spiked with the analytes or IS post-extraction, n=6. The % difference represents the deviation of extracted samples from neat standards. LOQ for Ru1 and Ru2, 0.03 µM; LQC for Ru3, 0.1 µM; HQC for Ru1 and Ru2, 0.5 µM; HQC for Ru3, 1.5 µM.

Complex	**Ru1**		**Ru2**		**Ru3**		**IS**
QC	LQC	HQC	LQC	HQC	LQC	HQC	
NS Mean ± SD	58484 ± 546	794353 ± 18774	198843 ± 2392	4524355 ± 121183	307119 ± 121183	5221194 ± 283685	40857 ± 1021
Cells Mean ± SD (% Difference[a])	58642 ± 703 (-0.27%)	784637 ± 47994 (+1.22%)	198465 ± 20878 (+ 0.19%)	4512173 ± 408373 (+ 0.27%)	309485 ± 11063 (- 0.77%)	5242925 ± 50379 (- 0.42%)	41096 ± 1365 (- 0.58%)
Plasma Mean ± SD (% Difference[a])	58900 ± 509 (-0.71%)	795506 ± 72041 (-0.15%)	197559 ± 6022 (+ 0.65%)	4520924 ± 126758 (+ 0.08%)	306623 ± 36055 (+ 0.16%)	5263393 ± 182086 (- 0.8%)	40952 ± 1373 (- 0.23%)
Urine Mean ± SD (% Difference[a])	59140 ± 6289 (-1.1%)	791219 ± 33884 (+0.39%)	200531 ± 15113 (- 0.85%)	4539302 ± 421029 (- 0.33%)	309511 ± 19951 (- 0.78%)	5272485 ± 264329 (- 0.98%)	40995 ± 1348 (- 0.34%)

NS, neat standard

[a] $\%\ Difference = \frac{Response\ of\ NS - Response\ of\ extracted\ sample}{Response\ of\ NS} \times 100$

Table S5 Intra-day and inter-day variability in the concentration of Ru2 measured by LC-MS/MS in cells (A), plasma (B), and urine (C). Quantification was based on calibration curves in Fig. S5. Six replicates were used at each QC and three independent runs were performed. LLOQ, 0.01 μM; LOQ, 0.03 μM; MQC, 0.1 μM, and HQC, 0.5 μM.

		Cells				Plasma				Urine			
	QC	**LLOQ**	**LQC**	**MQC**	**HQC**	**LLOQ**	**LQC**	**MQC**	**HQC**	**LLOQ**	**LQC**	**MQC**	**HQC**
Intra-day variability	**Mean ± SD (μM)**	0.0098 ± 0.0014	0.262 ± 0.0017	0.0907 ± 0.0075	0.435 ± 0.110	0.0084 ± 0.0010	0.0343 ± 0.0002	0.0991 ± 0.0078	0.430 ± 0.0133	0.0098 ± 0.0011	0.0340 ± 0.0030	0.0993 ± 0.0076	0.532 ± 0.0493
	Precision-RSD (%)	14.4	6.5	8.3	2.5	11.3	0.7	7.8	3.1	10.7	8.8	7.6	9.3
	Accuracy-RE[a] (%)	- 1.6	- 12.8	- 9.3	- 13.1	- 15.7	- 14.4	- 0.9	- 14.1	- 1.5	+ 13.3	- 0.7	+ 6.4
Inter-day variability (3 runs)	**Mean ± SD (μM)**	0.0106 ± 0.0006	0.0304 ± 0.0022	0.0935 ± 0.0074	0.5003 ± 0.0595	0.0110 ± 0.0014	0.0313 ± 0.0024	0.0963 ± 0.0024	0.471 ± 0.0350	0.0087 ± 0.0010	0.0290 ± 0.0043	0.0914 ± 0.0071	0.491 ± 0.0356
	Precision-RSD (%)	5.8	7.3	7.9	11.8	13.1	7.7	2.5	7.4	11.1	15.0	7.8	7.2
	Accuracy-RE[a] (%)	+ 5.7	+ 1.5	- 6.5	+ 0.59	+ 10.4	+ 4.2	- 3.7	- 5.7	- 12.6	- 3.4	- 8.6	- 1.7

[a] $RE = \frac{Mean\ calculated\ concentration - Nominal\ concentration}{Nominal\ concentration} \times 100$

Table S6 Intra-day and inter-day variability in the concentration of Ru3 measured by LC-MS/MS in cells (A), plasma (B), and urine (C). Quantification was based on calibration curves in Fig. S6. Six replicates were used at each QC and three independent runs were performed. LLOQ, 0.04 µM; LOQ, 0.1 µM; MQC, 0.5 µM, and HQC, 1.5 µM.

		Cells				Plasma				Urine			
	QC	**LLOQ**	**LQC**	**MQC**	**HQC**	**LLOQ**	**LQC**	**MQC**	**HQC**	**LLOQ**	**LQC**	**MQC**	**HQC**
Intra-day variability	**Mean ± SD (µM)**	0.0353 ± 0.0048	0.106 ± 0.0138	0.443 ± 0.0558	1.645 ± 0.1302	0.0467 ± 0.0018	0.104 ± 0.00194	0.532 ± 0.0201	1.453 ± 0.0412	0.0360 ± 0.0015	0.0855 ± 0.0035	0.434 ± 0.0628	1.455 ± 0.1775
	Precision-RSD (%)	13.5	13.0	12.6	7.9	3.8	1.9	3.8	2.8	4.1	4.1	14.5	12.2
	Accuracy-RE[a] (%)	- 11.7	+ 5.8	- 11.3	+ 9.7	+ 16.7	+ 4.0	+ 6.3	- 3.1	- 9.8	- 14.5	- 13.1	- 3.0
Inter-day variability (3 runs)	**Mean ± SD (µM)**	0.0384 ± 0.0028	0.0982 ± 0.0076	0.497 ± 0.0600	1.457 ± 0.1761	0.0464 ± 0.0008	0.110 ± 0.0052	0.545 ± 0.0149	1.520 ± 0.0717	0.0405 ± 0.0040	0.0945 ± 0.0087	0.518 ± 0.0727	1.570 ± 0.0992
	Precision-RSD (%)	7.2	7.7	12.0	12.1	1.7	4.7	2.7	4.7	9.9	9.2	14.0	6.3
	Accuracy-RE[a] (%)	- 3.9	- 1.8	- 0.51	- 2.8	+ 16.0	+ 9.8	+ 9.1	+ 1.3	+ 1.2	- 5.5	+ 3.5	+ 4.7

[a] $$RE = \frac{Mean\ calculated\ concentration - Nominal\ concentration}{Nominal\ concentration} \times 100$$

Table S7 Recovery of Ru2 from cells, plasma, and urine. Quantification was based on calibration curves represented in Fig. S5, and six replicates were used at each QC. LOQ, 0.03 μM; MQC, 0.1 μM, and HQC, 0.5 μM.

	LQC	MQC	HQC
Recovery from cells[a] (%) ± SD	85 ± 10	96 ± 9.9	91 ± 3.4
Recovery from plasma[a] (%) ± SD	101 ± 15	104 ± 11	95 ± 8.7
Recovery from urine[a] (%) ± SD	102 ± 11	99 ± 10	116 ± 22

[a] $Recovery = \frac{Mean\ concentration\ of\ extracted\ sample}{Mean\ concentration\ of\ the\ sample\ post\text{-}extraction} \times 100$

Table S8 Recovery of Ru3 from cells, plasma, and urine. Quantification was based on calibration curves represented in Fig. S6, and six replicates were used at each QC. LOQ, 0.1 μM; MQC, 0.5 μM, and HQC, 1.5 μM.

	LQC	MQC	HQC
Recovery from cells[a] (%) ± SD	93 ± 15	80 ± 13	112 ± 11
Recovery from plasma[a] (%) ± SD	98 ± 4.2	101 ± 5.1	102 ± 6.8
Recovery from urine[a] (%) ± SD	101 ± 8.0	97 ± 18	100 ± 15

[a] $Recovery = \frac{Mean\ concentration\ of\ extracted\ sample}{Mean\ concentration\ of\ the\ sample\ post\text{-}extraction} \times 100$

Table S9 Autosampler and freeze-thaw stability of Ru2. Extracted samples from cells, plasma, or urine were incubated in the autosampler for 18 h or exposed to four freeze-thaw cycles (from -80 ℃ to room temperature), before analysis. Quantification was based on calibration curves represented in Fig. S5, and three replicates were used at each QC level. LOQ, 0.03 μM and HQC, 0.5 μM.

		Cells		**Plasma**		**Urine**	
	QC	LQC	HQC	LQC	HQC	LQC	HQC
Autosampler stability	Mean ± SD (μM)	0.032 ± 0.001	0.490 ± 0.010	0.030 ± 0.001	0.507 ± 0.022	0.027 ± 0.001	0.478 ± 0.004
	RE[a] (%)	+ 6.0	- 2.1	- 0.01	+ 1.4	- 10.9	- 4.4
Freeze-thaw stability	Mean ± SD (μM)	0.030 ± 0.004	0.438 ± 0.029	0.029 ± 0.003	0.451 ± 0.028	0.033 ± 0.001	0.510 ± 0.058
	RE[a] (%)	+ 1.47	- 12.4	- 4.1	- 9.7	+ 9.5	+ 1.9

[a] $RE = \frac{Mean\ calculated\ concentration - Nominal\ concentration}{Nominal\ concentration} \times 100$

Table S10 Autosampler and freeze-thaw stability of Ru3. Extracted samples from cells, plasma, or urine were incubated in the autosampler for 18 h or exposed to four freeze-thaw cycles (from -80 ℃ to room temperature), before analysis. Quantification was based on calibration curves represented in Fig. S6, and three replicates were used at each QC level. LOQ, 0.1 μM and HQC, 1.5 μM.

		Cells		**Plasma**		**Urine**	
	QC	LQC	HQC	LQC	HQC	LQC	HQC
Autosampler stability	Mean ± SD (μM)	0.100 ± 0.010	1.516 ± 0.044	0.111 ± 0.003	1.537 ± 0.053	0.104 ± 0.003	1.537 ± 0.015
	RE[a] (%)	- 0.21	+ 1.1	+ 11.5	+ 2.5	+ 4.3	+ 2.4
Freeze-thaw stability	Mean ± SD (μM)	0.098 ± 0.012	1.375 ± 0.036	0.010 ± 0.002	1.542 ± 0.051	0.112 ± 0.015	1.276 ± 0.178
	RE[a] (%)	- 1.6	- 8.3	- 0.3	+ 2.8	+ 12.5	- 14.9

[a] $RE = \frac{Mean\ calculated\ concentration - Nominal\ concentration}{Nominal\ concentration} \times 100$

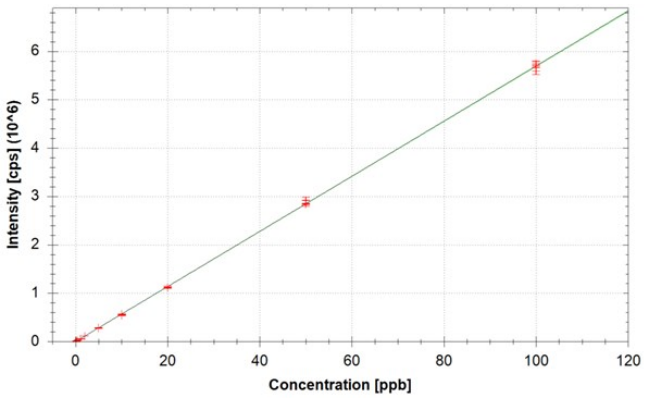

Fig. S15. Calibration curve for ^{102}Ru as measured by ICP-MS. The Ru standard solution (Sigma-Aldrich) was diluted with 2% HNO_3 to final concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, 1, 5, 10, 50, and 100 ppb, and indium was included as an internal standard (5 ppb). Calibration standards were acquired on Thermo Scientific iCAP RQ ICP-MS, using the Qtegra software. $y = 56937x + 66.7$, $r^2 = 0.9998$, lower limit of detection or LOD = 0.0027 ppb.