Bile Acid Profiling and Quantification in Biofluids using Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry

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ABSTRACT: Bile acids are important end products of cholesterol metabolism. While they have been identified as key factors in lipid emulsification and absorption due to their detergent properties, bile acids have also been shown to act as signaling molecules and intermediates between the host and the gut microbiota. To further the investigation of bile acid functions in humans, an advanced platform for high throughput analysis is essential. Herein we describe the development and application of a 15 minute UPLC chromatographic procedure for the separation of bile acid species from human biofluid samples requiring minimal sample preparation. High resolution time of flight mass spectrometry was applied for profiling applications, elucidating rich bile acid profiles in both normal and disease state plasma. In parallel, a second mode of detection was developed utilizing tandem mass spectrometry for sensitive and quantitative targeted analysis of 145 bile acid (BA) species including primary, secondary and tertiary bile acids. The latter system was validated by testing the linearity (LLOQ 0.25-10 nM and ULOQ 2.5-5 µM), precision (≈6.5%) and accuracy (81.2-118.9%) on inter- and intra-day analysis achieving good recovery of bile acids (serum/plasma 88% and urine 93%). The UPLC-MS/MS targeted method was successfully applied to plasma, serum, and urine samples in order to compare the bile acid pool compositional difference between pre-prandial and post-prandial states, demonstrating the utility of such analysis on human biofluids.

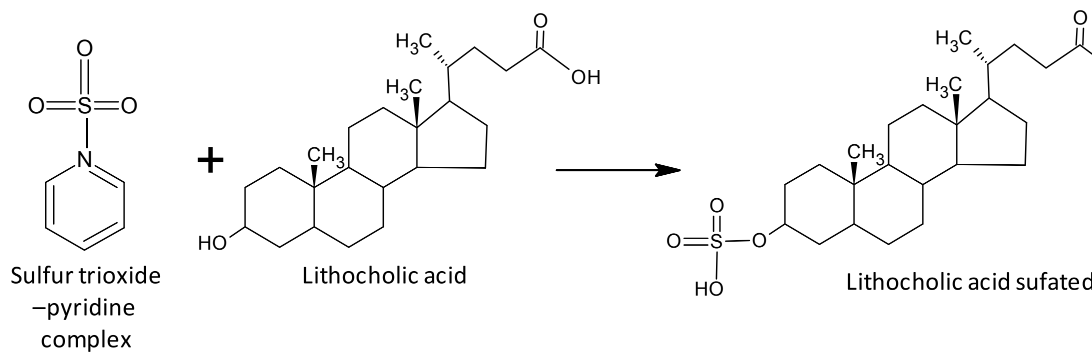

Figure S-1. Sulfation scheme with sulfur trioxide-pyridine complex. Example shown for lithocholic acid with sulfation on carbon position 3 in the steroid nucleus.

Table S-1. UPLC-MS/MS settings for the 57 bile acid standards, including 36 non-conjugated, 12 conjugated with taurine, 9 conjugated with glycine and 16 deuterated standards. Individual optimization of selected ion monitoring (SIM) and multiple reaction monitoring (MRM) transitions, collision energy (CE) and retention time (RT) was implemented. Abbreviations of BAs observed in Figure 1.

Numbers	Names and abbreviations	SIM *(m/z)*	CE (eV)	RT (min)
1	Ursocholanic Acid	359.2955	10	11.58
2	Lithocholenic Acid	373.2748	10	10.62
3	5-Cholenic Acid-3β-ol	373.2748	10	10.13
4	3-Ketocholanic Acid	373.2748	10	10.65
5	Isolithocholic Acid	375.2904	10	10.45
6	Allolithocholic Acid	375.2904	10	10.31
7	3α,12α, 23-Nordeoxycholic Acid	377.2697	10	9.55
8	9(11), (5β)-Cholenic Acid-3α-ol-12-one	387.2540	10	8.13
9	5α-Cholanic Acid-3, 6-dione	387.2540	10	7.76
10	3,7-Diketocholanic Acid	387.2540	10	7.83
11	3,6-Diketocholanic Acid	387.2540	10	8
12	3,12-Diketocholanic Acid	387.2540	10	8
13	8(14),(5β)-Cholenic Acid-3α, 12α-diol	389.2697	10	9.75
14	5β-Cholenic Acid-7α-ol-3-one	389.2697	10	9.81
15	5α-Cholanic Acid-3α-ol-6-one	389.2697	10	8.13
16	3α-Hydroxy-7 Ketolithocholic Acid	389.2697	10	8.45
17	3α-Hydroxy-12 Ketolithocholic Acid	389.2697	10	8.91
18	Lithocholic acid	375.2904	10	10.78
19	5β-Cholanic Acid-3β, 12α-diol	391.2853	10	8.93
20	Chenodeoxycholic Acid (CDCA)	391.2853	10	10.22
21	Deoxycholic Acid (DCA)	391.2853	10	10.33
22	Hyodeoxycholic acid	391.2853	10	8.66
23	Isodeoxycholic Acid	391.2853	10	10.57
24	Murocholic Acid (MuroCA)	391.2853	10	7.33
25	Ursodeoxycholic acid (UDCA)	391.2853	10	8.13
26	3,7,12 Dehydrocholic acid	401.2333	10	3.75
27	3α-Hydroxy-7,12-DiketoCholanic Acid	403.2489	10	4.38
28	3α-Hydroxy-6,7-DiketoCholanic Acid	403.2489	10	8.21
29	5β-Cholanic Acid-3α, 6α-diol-7-one	405.2646	10	6.64
30	3 Dehydrocholic Acid	405.2646	10	7.26
31	12 Dehydrocholic Acid	405.2646	10	6.18
32	α Muricholic Acid	407.2802	10	6.64
33	β Muricholic Acid	407.2802	10	6.72
34	ω Muricholic Acid	407.2802	10	6.52
35	Cholic acid (CA)	407.2802	10	8.38
36	Hyocholic acid (HCA)	407.2802	10	7.83

Numbers	Names and abbreviations	MRM transitions and SIM *(m/z)*	CE (eV)	RT (min)
37	Glyco-ursocholanic Acid	416.3170 → 74	40	11.1
38	Glycolithocholic Acid (GLCA)	432.3119 → 74	40	9.86
39	Glycoursodeoxycholic Acid (GUDCA)	448.3068 → 74	40	5.23
40	Glycohyodeoxycholic Acid (GHDCA)	448.3068 → 74	40	5.6
41	Glycochenodeoxycholic Acid (GCDCA)	448.3068 → 74	40	7.67
42	Glycodeoxycholic acid (GDCA)	448.3068 → 74	40	8.14
43	3,7,12 Glycodehydrocholic acid	458.2548 → 74	40	1.76
44	Glycocholic Acid (GCA)	464.3017 → 74	40	5.85
45	Glycohyocholic Acid (GHCA)	464.3017 → 74	40	4.89
46	Taurolithocholic Acid (TLCA)	466.2996 → 80	40	9.03
47	Tauro-ursocholanic Acid	482.2945 → 80	60	9.15
48	Tauro-ursodeoxycholic Acid (TUDCA)	498.2894 → 80	60	4.68
49	Taurohyodeoxycholic Acid	498.2894 → 80	60	5
50	Taurochenodeoxycholic Acid (TCDCA)	498.2894 → 80	60	7
51	Taurodeoxycholic Acid (TDCA)	498.2894 → 80	60	7.45
52	3,7,12 Taurodehydrocholic acid	508.2374 → 80	60	1.45
53	Taurohyocholic Acid (THCA)	514.2843 → 80	60	4.32
54	Tauro-β Muricholic Acid	514.2843 → 80	60	3.22
55	Tauro-α Muricholic Acid	514.2843 → 80	60	3.22
56	Tauro ω-Muricholic Acid (TωM)	514.2843 → 80	60	3.18
57	Taurocholic Acid (TCA)	514.2843 → 80	60	5.2
58	Lithocholic Acid-d4	379.5893	10	10.81
59	Deoxycholic Acid-13C	395.5886	10	10.34
60	Chenodeoxycholic Acid-d4	395.5886	10	10.21
61	Ursodeoxycholic Acid-d4	395.5886	10	8.21
62	Hyodeoxycholic Acid-d5	396.5949	10	8.64
63	Cholic Acid-d4	411.5881	10	8.98
64	Glycolithocholic Acid-d4	436.6406 → 74	40	9.96
65	Glycochenodeoxycholic Acid-d4	451.5321 → 74	40	7.56
66	Glycodeoxycholic Acid-d4	452.6400 → 74	40	8.28
67	Glycoursodeoxycholic Acid-d4	452.6400 → 74	40	5.37
68	Glycocholic Acid-d4	468.6394 → 74	40	7.9
69	Taurolithocholic Acid-d4	486.7209 → 80	60	8.75
70	Taurochenodeoxycholic Acid-d4	502.7203 → 80	60	7.28
71	Tauroursodeoxycholic Acid-d5	503.7265 → 80	60	4.93
72	Taurocholic Acid-d4	518.7197 → 80	60	5
73	Taurodeoxycholic Acid-d4	501.7124 → 80	60	7.15

Table S-2. UPLC-MS/MS settings for mono-sulfated bile acid standards sorted according to sulfation ratio and retention time, including; 44 BAs sulfated in position OH-C3, 15 sulfated in position OH-C6, 21 sulfated in position OH-C7 and 8 sulfated in position OH-C12 sulfated. Individual optimization of multiple reaction monitoring (MRM) transitions, collision energy (CE) and retention time (RT) was implemented. Abbreviations of BAs observed in Figure 1.

BA number	Names an Abbreviations	OH group	MRM transitions (*m/z*)	CE (eV)	a	b	c
74	Lithocholenic acid	3-OH	453.2316 → 97	40	8.74		
75	5-Cholenic Acid-3β-ol	3-OH	453.2316 → 97	40	7.73		
76	Isolithocholic Acid	3-OH	455.2473 → 97	40	8.44		
77	Allolithocholic Acid	3-OH	455.2473 → 97	40	8.15		
78	Lithocholic acid (LCA-S)	3-OH	455.2473 → 97	40	9.53		
79	3α,12α, 23-Nordeoxycholic Acid	3-OH; 12-OH	457.2265 → 97	40	6.4	7.11	
80	9(11), (5β)-Cholenic Acid-3α-ol-12-one	3-OH	467.2108 → 97	40	6.48		
81	5β-Cholenic Acid-7α-ol-3-one	3-OH	469.2265 → 97	40	7.1		
82	5α-Cholanic Acid-3α-ol-6-one	3-OH	469.2265 → 97	40	5.92		
83	3α-Hydroxy-7 Ketolithocholic Acid	3-OH	469.2265 → 97	40	6		
84	3α-Hydroxy-12 Ketolithocholic Acid	3-OH	469.2265 → 97	40	6.04		
85	8(14),(5β)-Cholenic Acid-3α, 12α-diol	3-OH; 12-OH	469.2265 → 97	40	6.58	7.49	
86	Murocholic Acid	3-OH; 6-OH	471.2421 → 97	40	4.9	5.13	
87	Isodeoxycholic Acid	7-OH; 12-OH	471.2421 → 97	40	7.8	8.06	
88	5β-Cholanic Acid-3β, 12α-diol	3-OH; 6-OH	471.2421 → 97	40	6.66	6.36	
89	Chenodeoxycholic Acid	3-OH; 7-OH	471.2421 → 97	40	7.67	7.96	
90	Hyodeoxycholic acid	3-OH; 6-OH	471.2421 → 97	40	5.62	6.48	
91	Ursodeoxycholic acid	3-OH; 7-OH	471.2421 → 97	40	5.48	6.53	
92	Deoxycholic Acid	3-OH; 12-OH	471.2421 → 97	40	7.89	8.22	
93	3α-Hydroxy-7,12-DiketoCholanic Acid	3-OH	483.2058 → 97	40	2.34		
94	3α-Hydroxy-6,7-DiketoCholanic Acid	3-OH	483.2058 → 97	40	5		
95	5β-Cholanic Acid-3α, 6α-diol-7-one	3-OH; 6-OH	485.2214 → 97	40	4.65	4.26	
96	3 Dehydrocholic Acid	7-OH; 12-OH	485.2214 → 97	40	4.16	-	
97	12 Dehydrocholic Acid	3-OH; 7-OH	485.2214 → 97	40	3.72	3.45	
98	α Muricholic Acid	3-OH; 6-OH; 7-OH	487.2371 → 97	40	4.17	4.04	5.51
99	ω Muricholic Acid	3-OH; 6-OH; 7-OH	487.2371 → 97	40	4.42	5.93	4.6
100	β Muricholic Acid	3-OH; 6-OH; 7-OH	487.2371 → 97	40	4.68	5.29	4.34
101	Cholic acid	3-OH; 7-OH; 12-OH	487.2371 → 97	40	5.84	5.54	4.84
102	Hyocholic acid	3-OH; 6-OH; 7-OH	487.2371 → 97	40	4.66	5.95	5.17
103	Glycolithocholic Acid	3-OH	512.2687 → 97	40	6.78		
104	Glycoursodeoxycholic Acid	3-OH; 7-OH	528.2636 → 97	40	3.05	4.11	
105	Glycohyodeoxycholic Acid	3-OH; 6-OH	528.2636 → 97	40	3.01	3.85	
106	Glycochenodeoxycholic Acid (GCDCA-S)	3-OH; 7-OH	528.2636 → 97	40	4.66	5.17	
107	Glycodeoxycholic acid	3-OH; 12-OH	528.2636 → 97	40	5.48	4.77	
108	Glycocholic Acid	3-OH; 7-OH; 12-OH	566.2405 → 97	40	5.95	5.87	5.67
109	Glycohyocholic Acid	3-OH; 6-OH; 7-OH	566.2405 → 97	40	2.74	2.31	3.56
110	Taurolithocholic acid	3-OH	562.2513 → 97	40	5.29		
111	Tauro-ursodeoxycholic Acid	3-OH; 7-OH	578.2462 → 97	40	2.26	3.14	
112	Taurohyodeoxycholic Acid	3-OH; 6-OH	578.2462 → 97	40	2.2	2.92	
113	Taurochenodeoxycholic Acid	3-OH; 7-OH	578.2462 → 97	40	4.01	4.28	
114	Taurodeoxycholic Acid	3-OH; 12-OH	578.2462 → 97	40	3.59	4.02	
115	Taurohyocholic Acid	3-OH; 6-OH; 7-OH	594.2412 → 97	40	1.55	1.91	2.44
116	Tauro-β Muricholic Acid	3-OH; 6-OH; 7-OH	594.2412 → 97	40	1.59	1.34	2.03
117	Tauro-α Muricholic Acid	3-OH; 6-OH; 7-OH	594.2412 → 97	40	1.51	1.61	2.27
118	Tauro ω-Muricholic Acid	3-OH; 6-OH; 7-OH	594.2412 → 97	40	1.57	1.51	2
119	Taurocholic Acid	3-OH; 7-OH; 12-OH	594.2412 → 97	40	2.63	2.68	-

Table S-3. Chromatographic gradient used for BA profiling and targeted analysis.

Time(min)	Flow Rate	%A	%B	Curve
Initial	0.6	90	10	Initial
0.1	0.6	90	10	6
9.25	0.6	65	35	6
11.5	0.65	15	85	6
11.8	0.8	0	100	6
12	0.95	0	100	6
12.1	1	0	100	6
12.4	1	0	100	6
12.45	0.85	45	55	6
12.5	0.85	90	10	6
12.6	0.8	90	10	6
12.7	0.7	90	10	6
12.8	0.6	90	10	6
15.0	0.6	90	10	6

Table S-4. Percent yield of the 16 reactions (3 replicates) of lithocholic acid (LCA) modified into sulfated lithocholic acid (LCA-S).

	Sulfation conditions															
	$CHCl_3$								**Pyridine**							
	1h	**24h**	**1h**	**24h**	**1h**	**24h**	**1h**	**24h**	**1h**	**24h**	**1h**	**24h**	**1h**	**24h**	**1h**	**24h**
	RT	**RT**	**55°C**	**55°C**	**RT**	**RT**	**55°C**	**55°C**	**RT**	**RT**	**55°C**	**55°C**	**RT**	**RT**	**55°C**	**55°C**
					Sodium sulfate								**Sodium sulfate**			
LCA	96.1	95.8	79.9	6.4	92.2	89.8	83.5	17.2	43.4	49.0	40.3	47.5	41.2	47.1	47.4	44.2
LCA-S	3.9	4.2	20.1	93.6	7.8	10.2	16.5	82.8	56.6	51.0	59.7	52.5	58.8	52.9	52.6	55.8
standard deviation	3.05	3.2	6.34	2.09	5.38	5.88	6.47	10.7	7.86	9.69	4.69	10.5	8.33	3.1	8.42	22.2

Table S-5. Intra- and inter-day validation of accuracy and precision over 57 bile acids with QC1 (10nM), QC2 (50nM), QC3 (100nM) and QC4 (750nM). Limit of detection (LOD), lower limit of quantification (LLOQ), upper limit of quantification and R^2

					INTRA DAY								INTER DAY							
					Accuracy %				Precision %				Accuracy %				Precision %			
	LOD (nM)	LLOQ (nM)	ULOQ (µM)	R^2	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4
1	0.1	0.25	2.5	0.9978	104.0	100.9	102.1	101.9	1.3	4.4	1.8	3.8	100.2	99.5	100.4	96.1	6.3	3.8	3.7	0.9
2	0.5	0.75	5	0.999	108.7	101.9	103.0	102.8	5.8	3.1	6.8	6.7	93.2	97.4	99.9	96.5	13.3	3.8	8.0	2.6
3	0.75	1	5	0.998	106.1	100.9	98.4	105.3	1.9	3.6	1.6	2.3	96.6	99.2	104.5	97.5	13.6	3.9	4.9	12.5
4	0.05	0.25	2.5	0.9985	111.8	98.1	101.2	104.4	5.1	5.5	3.2	1.9	114.1	100.9	102.0	99.3	6.0	8.9	4.6	9.7
5	0.25	0.5	5	0.999	89.5	98.7	99.9	106.1	5.3	4.3	1.3	4.1	81.2	98.7	100.9	96.7	10.1	5.7	3.7	0.2
6	0.25	0.5	5	0.999	108.8	102.1	102.8	104.4	1.4	4.6	2.0	2.0	87.4	100.9	104.4	96.5	10.9	5.0	6.3	8.2
7	0.25	0.5	2.5	0.999	103.7	98.9	100.4	105.8	2.3	4.2	1.8	3.0	101.3	99.4	102.7	99.5	7.3	5.6	7.5	10.8
8	0.5	0.75	2.5	0.9994	114.7	102.3	106.2	104.1	4.6	4.8	1.5	1.6	108.0	106.2	109.4	98.9	14.1	6.6	3.0	8.4
9	0.5	0.75	2.5	0.9999	105.7	100.6	108.1	104.3	5.3	4.6	1.6	2.0	102.8	111.1	110.0	99.2	6.7	8.0	4.8	8.3
10	0.25	0.5	5	0.9991	94.7	101.8	101.3	103.8	14.0	3.1	3.7	2.8	103.4	102.0	104.1	101.8	8.5	8.0	5.5	0.0
11	0.5	0.75	5	0.997	85.7	96.8	94.2	107.1	7.7	6.8	3.4	3.2	85.7	98.3	95.5	98.3	15.5	8.0	5.6	0.4
12	0.5	0.75	5	0.9997	106.5	98.6	100.0	107.1	12.4	7.0	4.2	3.2	102.6	95.6	95.5	98.3	19.3	9.5	5.1	0.4
13	2.5	5	5	0.999	92.7	95.1	97.2	104.4	3.2	4.0	1.4	3.7	92.9	100.4	103.6	94.8	13.1	4.7	8.4	5.3
14	0.25	0.5	2.5	0.9999	117.1	97.7	99.7	103.5	2.3	4.9	1.8	2.4	118.2	101.3	106.5	98.0	2.1	8.4	4.8	9.6
15	1	2.5	5	0.9985	87.4	100.4	101.4	106.1	6.4	4.0	2.6	3.4	90.9	91.5	90.7	98.9	13.4	14.4	7.4	6.1
16	1	2.5	2.5	0.9999	108.4	97.5	98.7	103.2	2.8	5.1	2.3	3.2	117.3	103.2	106.4	97.5	2.5	6.5	5.0	11.2
17	2.5	5	5	0.999	104.7	102.9	100.7	105.6	3.7	3.1	1.2	2.9	96.7	98.6	102.5	96.6	8.9	5.8	7.7	7.5
18	0.25	0.5	5	0.9953	101.7	99.9	100.5	112.1	5.1	4.2	3.7	12.4	99.6	96.9	92.3	98.4	18.8	11.3	5.3	21.7
19	0.25	0.5	2.5	0.9982	104.3	99.2	102.4	103.5	2.1	4.7	2.0	1.8	111.7	101.2	105.6	97.6	3.3	9.1	4.2	11.2
20	1	2.5	5	0.9999	95.2	97.6	99.2	102.4	2.5	4.9	1.4	8.2	101.3	99.4	102.7	74.9	7.3	5.6	7.5	31.6
21	0.25	0.5	2.5	0.9991	107.4	99.3	104.6	103.3	2.5	4.6	1.9	2.7	103.6	103.1	107.9	97.0	10.3	2.7	4.1	7.6
22	0.25	0.5	5	0.9999	105.8	97.9	99.7	105.3	2.0	5.1	2.2	2.5	114.3	103.8	106.9	95.4	3.8	8.0	4.8	2.6
23	0.25	0.5	5	0.9998	101.3	96.8	97.8	104.4	8.1	5.0	1.5	5.6	109.2	100.2	103.7	98.4	11.2	5.9	8.1	1.0
24	0.25	0.5	2.5	0.9905	95.3	100.2	101.8	103.4	7.4	4.1	2.5	3.8	104.6	94.5	91.1	98.2	11.3	16.6	10.6	11.4
25	0.5	1	5	0.9958	93.7	98.8	99.2	102.9	4.5	4.8	3.2	6.7	99.5	94.8	90.3	102.5	13.9	11.7	10.7	3.0
26	0.25	0.5	5	0.9934	96.9	100.7	105.0	105.2	5.1	4.4	4.7	2.3	101.5	95.1	92.4	99.6	13.7	15.4	8.8	9.7

					INTRA DAY								INTER DAY							
	LOD (nM)	LLOQ (nM)	ULOQ (μM)	R2	Accuracy %				Precision %				Accuracy %				Precision %			
					QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4
27	0.25	0.5	2.5	0.9999	95.3	103.7	97.0	103.4	3.9	2.9	1.5	2.0	102.9	98.5	102.2	99.4	9.4	5.0	9.0	6.7
28	0.25	0.5	5	0.9998	89.6	101.8	102.0	103.3	9.7	4.2	1.5	2.4	96.0	102.9	103.5	97.8	19.0	2.6	4.4	6.2
29	0.25	0.5	2.5	0.999	100.1	100.8	102.2	104.9	3.0	4.1	1.5	7.1	96.8	101.2	104.5	105.7	9.5	3.6	6.1	0.1
30	0.1	0.25	5	0.999	102.1	103.6	105.8	107.0	1.0	4.1	1.6	3.9	96.8	100.3	104.9	97.7	9.1	5.5	7.1	4.6
31	2.5	5	5	0.9988	91.3	89.4	98.3	106.0	8.8	5.9	1.9	2.7	100.3	101.6	104.0	96.9	13.5	6.3	5.7	12.1
32	0.5	0.75	5	0.9998	93.5	100.1	101.6	103.3	3.0	4.4	2.4	3.8	97.0	102.5	105.5	101.7	11.1	5.0	6.9	0.1
33	0.5	0.75	2.5	0.9997	94.2	99.0	100.9	106.1	3.3	3.6	1.5	2.2	96.1	100.8	104.1	99.4	11.5	4.9	6.8	11.8
34	0.5	0.75	2.5	0.9999	101.4	101.3	100.7	106.3	2.6	4.1	1.8	2.0	103.7	92.7	93.3	100.1	6.0	10.3	9.2	11.0
35	0.5	0.75	5	0.9995	88.6	88.6	98.6	104.0	11.5	6.3	1.8	4.6	93.3	102.0	105.0	103.1	16.1	6.7	5.8	1.7
36	0.75	1	5	0.9998	99.3	105.7	106.9	105.0	4.0	2.2	4.9	3.4	105.5	100.1	101.3	94.6	14.1	5.3	9.0	4.3
37	7.5	10	5	0.9915	104.4	108.4	103.1	106.4	6.6	4.3	5.4	4.2	103.6	102.4	104.5	96.2	10.2	7.1	5.7	5.2
38	5	7.5	5	0.9998	81.5	97.9	111.0	107.2	7.9	6.6	1.0	9.0	92.3	100.6	105.5	94.9	13.4	6.9	5.2	14.4
39	2.5	5	5	0.9998	108.9	108.4	107.0	102.1	9.3	4.3	2.1	3.5	112.6	101.1	110.1	101.3	14.3	13.3	4.7	3.3
40	2.5	5	5	0.9998	100.9	97.2	97.8	105.7	11.1	4.8	1.5	2.6	115.8	99.7	101.6	99.0	6.0	4.9	7.9	12.6
41	2.5	5	5	0.9995	106.7	103.5	110.0	105.1	15.2	3.8	6.6	5.2	118.9	92.8	102.9	95.5	12.9	5.6	11.3	14.5
42	2.5	5	2.5	0.9998	104.6	103.0	96.5	107.5	5.3	3.1	4.4	2.8	108.3	99.2	103.9	99.6	7.9	3.7	6.4	10.7
43	5	7.5	5	0.9998	91.1	95.8	112.9	101.4	15.5	6.0	1.4	1.2	101.4	102.1	105.8	94.9	19.6	5.9	5.2	14.9
44	5	7.5	5	0.9995	96.4	96.2	98.9	102.1	8.3	4.7	5.4	7.2	98.8	102.3	102.9	95.1	14.8	5.2	8.1	9.2
45	2.5	5	5	0.9998	100.5	102.9	107.7	102.6	7.6	6.2	2.7	2.2	107.4	108.4	112.4	101.2	11.1	6.9	3.8	2.1
46	2.5	5	5	0.9993	105.5	101.9	104.3	105.6	9.6	4.9	4.6	7.1	105.4	103.8	94.9	98.0	13.4	6.6	8.5	3.2
47	5	7.5	5	0.9998	104.5	98.7	98.9	103.4	8.7	4.6	1.7	18.3	95.4	97.9	104.6	99.4	8.8	4.7	6.1	6.7
48	2.5	5	2.5	0.9958	93.2	94.0	104.6	103.3	7.5	6.9	1.7	20.8	102.5	102.8	104.5	97.8	18.8	7.7	4.5	6.2
49	2.5	5	2.5	0.9996	106.4	99.0	98.1	104.9	8.3	4.6	3.7	2.3	95.9	100.3	106.8	105.7	10.3	2.7	4.6	0.1
50	2.5	5	5	0.9997	97.2	98.7	100.3	107.0	14.2	5.5	6.4	2.0	106.2	102.3	94.2	97.7	16.4	6.0	7.8	4.6
51	5	7.5	5	0.9978	97.2	93.9	106.5	106.0	10.8	6.8	1.9	2.4	91.9	107.1	109.9	96.9	15.0	7.9	3.1	12.1
52	2.5	5	5	0.9999	106.8	99.7	103.9	103.3	4.9	2.7	4.9	7.1	114.6	102.1	109.9	101.7	9.3	4.2	5.8	0.1
53	2.5	5	5	0.9996	106.2	108.2	113.7	101.5	3.8	7.4	1.2	2.3	86.7	115.7	118.1	101.4	11.3	8.8	3.4	0.7
54	2.5	5	5	0.9999	104.9	100.0	100.4	105.6	7.1	6.1	2.9	2.5	101.8	101.1	105.2	98.6	7.9	2.3	5.8	4.5
55	2.5	5	5	0.999	95.2	99.3	99.2	101.3	12.4	3.9	2.9	1.4	113.8	102.3	105.9	101.1	19.9	6.5	3.2	1.3
56	2.5	5	5	0.9985	97.2	97.8	92.6	91.7	14.9	4.6	3.1	15.1	103.5	91.4	89.3	88.8	18.0	8.1	6.0	31.1
57	0.5	1	5	0.9959	103.3	112.3	105.7	101.1	15.1	3.1	5.8	0.8	82.1	105.7	106.7	98.2	19.2	7.4	4.1	7.2

Table S-6. Quantitative matrix effect was evaluated by comparing integrated peak area expressed as a percentage of deuterated standards spiked in solvent and spiked in plasma samples for the QC1 (10 nM) and QC3 (100 nM) (n=3).

Labelled standards	QC1	QC3
58	96.19	80.70
59	98.07	81.25
60	107.81	92.22
61	88.99	85.03
62	93.83	80.61
63	91.55	81.03
64	97.09	83.86
65	91.43	111.37
66	106.70	84.46
67	96.80	85.35
68	111.85	87.09
69	91.70	79.85
70	87.05	83.80
71	105.45	86.76
72	94.22	83.06
73	90.38	93.27

Table S-7. Carry-over was evaluated on the integrated area under the peak of each labelled deuterated standards with the following run sequence; Blank 1 was injected first then QC1 (10nM), QC5 (5µM) and Blank 2.

Labelled standards	**Blank 2 *vs* QC5 %**	**Blank 2 *vs* QC1 %**	**Blank 1 *vs* QC1 %**
58	0.51	1.94	0.10
59	0.85	4.01	2.35
60	0.08	0.37	0.19
61	0.09	2.24	0.46
62	0.12	0.66	0.29
63	0.09	1.22	0.03
64	0.07	0.40	0.28
65	0.09	0.56	0.07
66	0.02	0.11	0.08
67	0.07	0.38	0.15
68	0.12	0.70	0.16
69	0.08	1.96	0.17
70	0.19	0.97	0.13
71	0.07	0.39	0.16
72	0.04	0.81	0.13
73	0.02	0.13	0.23

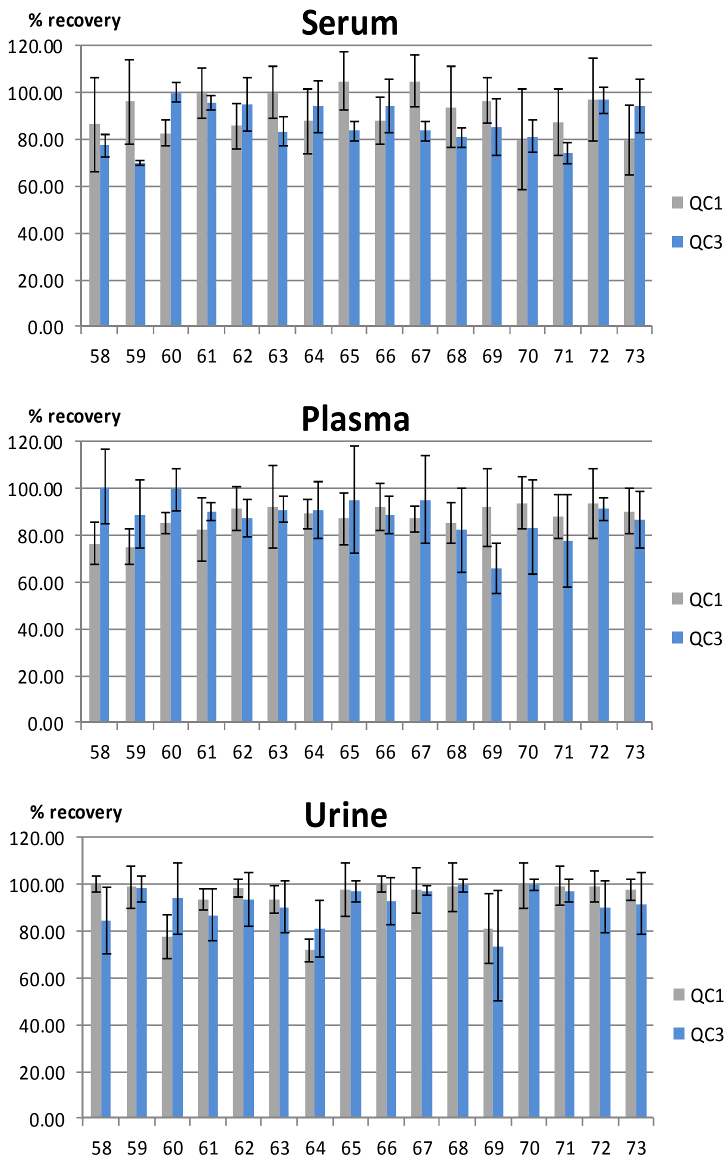

Figure S-2. Recoveries of the 16 deuterated standards evaluated at QC1 (10 nM) and QC3 (100 nM) in human plasma, serum and urine. The error bars show the standard deviation of the replicates recovery (n=6).

Table S-8. Recoveries and standard deviations (SD) of 16 internal labelled standards evaluated at QC1 (10 nM) and QC3 (100 nM) for human plasma, serum and urine (n=6).

		Serum		Plasma		Urine	
Labelled standards	**QC**	**Recoveries %**	**SD**	**Recoveries %**	**SD**	**Recoveries %**	**SD**
58	**QC1**	86.31	21.55	76.31	8.03	99.82	1.00
	QC3	77.27	5.93	100.52	41.67	84.44	23.35
59	**QC1**	96.01	23.14	75.06	15.15	98.61	4.62
	QC3	69.81	9.10	88.88	18.20	98.11	2.67
60	**QC1**	82.67	14.02	85.09	11.87	77.29	12.45
	QC3	99.98	6.61	99.32	5.02	93.86	12.56
61	**QC1**	99.63	10.41	82.38	14.71	93.38	5.64
	QC3	95.35	4.48	90.09	4.65	86.79	10.01
62	**QC1**	85.54	11.32	91.31	8.73	98.26	4.18
	QC3	95.00	11.91	87.31	8.66	93.27	11.30
63	**QC1**	99.82	10.65	92.01	18.63	93.37	7.35
	QC3	83.33	7.83	90.91	6.61	90.15	10.75
64	**QC1**	87.73	6.34	88.92	8.35	71.69	11.30
	QC3	93.85	5.07	90.61	8.93	81.14	13.22
65	**QC1**	104.68	21.42	87.04	12.20	97.38	8.93
	QC3	83.45	7.09	95.05	19.58	97.03	3.75
66	**QC1**	87.76	10.10	92.19	10.07	99.86	3.65
	QC3	94.34	11.35	88.57	8.18	92.88	10.02
67	**QC1**	104.68	18.32	87.04	10.15	97.38	7.66
	QC3	83.45	4.18	95.05	13.87	97.03	1.88
68	**QC1**	93.61	13.13	85.14	6.08	98.62	12.98
	QC3	80.61	4.07	82.04	16.54	99.45	2.82
69	**QC1**	96.47	9.00	91.91	16.73	80.81	18.52
	QC3	84.94	4.66	65.72	15.50	73.64	21.61
70	**QC1**	79.75	13.88	93.58	12.20	99.32	6.00
	QC3	81.15	4.90	83.27	19.37	99.77	1.95
71	**QC1**	87.17	19.13	87.93	9.62	99.10	6.75
	QC3	74.17	3.22	77.54	15.32	97.11	4.94
72	**QC1**	96.65	15.88	93.40	15.28	99.10	6.74
	QC3	96.63	5.62	91.00	5.53	90.08	10.64
73	**QC1**	79.51	14.76	90.22	9.51	97.53	4.75
	QC3	94.06	11.23	86.32	11.95	91.56	13.12