Application Note

Characterization of Human Plasma as a Reference Material for Lipid Analysis Using LC-MS

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Key Features

- A preparation of human plasma has been characterized as a reference material for lipid identification and quantitation using LC-MS.
- The validity of the methods used and the values obtained is supported by the reasonable agreement with published values in NIST SRM 1950 plasma.
- MaxSpec® Reference Plasma (Human) can be used for quality control, method development, and performance validation in lipid studies.
- A list of lipid concentrations is made public and periodically updated.

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Introduction

Lipids are key molecules for maintaining cellular membranes, energy storage, cellular signaling, and many other biological processes. The identification and understanding of their diverse functions are useful for insights into cell biology, metabolism, and disease mechanisms. Mass spectrometry has become the standard analytical tool for the identification and quantitation of hundreds of lipids, helping to reveal their roles in biological systems.

The use of well-characterized reference materials increases confidence in lipid quantitative data by validating the analytical methods employed. The objective of this study is to characterize a preparation of human plasma so it can be made available as reference material for the analysis of a wide variety of lipids using targeted methods for the analysis of oxylipins and bile acids, as well as untargeted lipidomics methods. All methods used in this characterization are validated by comparison of the obtained values using the well-established NIST SRM 1950 Metabolites in Human Plasma material to the published values.[1,2]

Experimental Procedures

Figure 1. Workflow of lipid extraction and analysis of plasma using LC-MS.

Replicate 50 µl aliquots (three for each analytical method) of each plasma (MaxSpec® or NIST SRM 1950) were extracted after mixtures of deuterated internal standards were added (See Table 1 for internal standard list). Acetonitrile extraction was used to precipitate proteins and isolate bile acids.[3] Oxylipins were isolated using reversed-phase polymeric sorbent solid-phase extraction (SPE).[4] For untargeted analysis, methyl-*tert*-butyl ether-based extraction was used.[5] Separation and quantitation of bile acids and oxylipins were achieved by reversed-phase LC-MS/MS using an Exion UPLC system coupled with a triple-quadrupole 6500+ mass spectrometer (Sciex). Separation and quantitation of all lipids using an untargeted approach were achieved using reversed-phase HPLC coupled with a high-resolution Q Exactive™ Orbitrap™ mass spectrometer (Thermo Fisher Scientific).

Table 1. Deuterated internal standards used for the three types of analysis. [1]Components of Deuterated Bile Acids MaxSpec® Discovery Mixture; Item No. 33506. [2]Components of Deuterated Lipidomics MaxSpec® Mixture; Item No. 40974.

Oxylipins	Item No.	Bile Acids[1]	Item No.	Untargeted[2]	Item No.
PGE_2-d_4	10007273	Cholic Acid-d_4	31348	FA(16:0-d_5)	30557
TXB_2-d_4	319030	Taurocholic Acid-d_4	31375	CAR(17:0)-d_3	9003263
5-HETE-d_8	334230	Glycocholic Acid-d_4	31352	DG(16:0-d_9/16:0)	27591
LTB_4-d_4	320110	Deoxycholic Acid-d_4	31350	TG(16:0-d_9/16:0/16:0)	27592
LTD_4-d_5	10006199	Taurodeoxycholic Acid-d_4	31563	PC(16:0-d_9/16:0)	28154
LXA_4-d_5	10007737	Glycodeoxycholic Acid-d_4	31553	LPC(16:0-d_9)	28153
14(15)-EET-d_{11}	10006410	Chenodeoxycholic Acid-d_4	31366	PE(16:0-d_9/16:0)	28155
14(15)-DiHET-d_{11}	10008040	Taurochenodeoxycholic Acid-d_4	31362	LPE(16:0-d_9)	27588
9-HODE-d_4	338410	Glycochenodeoxycholic Acid-d_4	31364	PG(16:0-d_9/16:0)	28953
RvE1-d_4	10009854	Lithocholic Acid-d_4	31354	PI(16:0-d_9/16:0)	28156
RvD1-d_5	11182	Taurolithocholic Acid-d_4	31571	PS(16:0-d_9/16:0)	28152
12,13-DiHOME-d_4	10009994	Glycolithocholic Acid-d_4	31554	Cer(18:1(4E)-d_7;1OH,3OH/16:0)	22787
13,14-Dihydro-15kPGD$_2$-d_9	19334	Ursodeoxycholic Acid-d_4	31368	SM(18:1(4E)-;1OH,3OH/16:0-d_9)	27551
15-HETE-d_8	334720	Tauroursodeoxycholic Acid-d_4	31564	Cholesterol-d_7	25265
PGA_2-d_4	310210	Glycoursodeoxycholic Acid-d_4	31555	CE(16:0-d_9)	28123

To quantitate bile acids and oxylipins, the integrated area of the LC-MS/MS signal corresponding to each analyte was normalized to the integrated area of its corresponding internal standard, when possible, or a surrogate internal standard. Using MultiQuant software (Sciex), these area ratios were interpolated into an authentic or surrogate calibration curve to provide a calculated concentration value. Untargeted lipidomics data was analyzed using Lipostar software for feature detection, peak alignment, normalization, and lipid identification. The integrated areas corresponding to all detected analytes were also normalized to the integrated areas corresponding to internal standards within their class, when possible, and single-point calibration using the known amount of internal standard added to samples was used to calculate the concentrations of all analytes. In this initial study, only those lipid molecular species for which published data were available for validation using the NIST plasma were quantified.

Results

Analysis of the NIST SRM 1950 plasma and a comparison of the data obtained with published NIST SRM values is a necessary initial step to validate the methods used to characterize the MaxSpec® plasma.[1,2] As shown in Figure 2 and Table 2, the lipid analytical data compare well with published reference values for the NIST plasma.

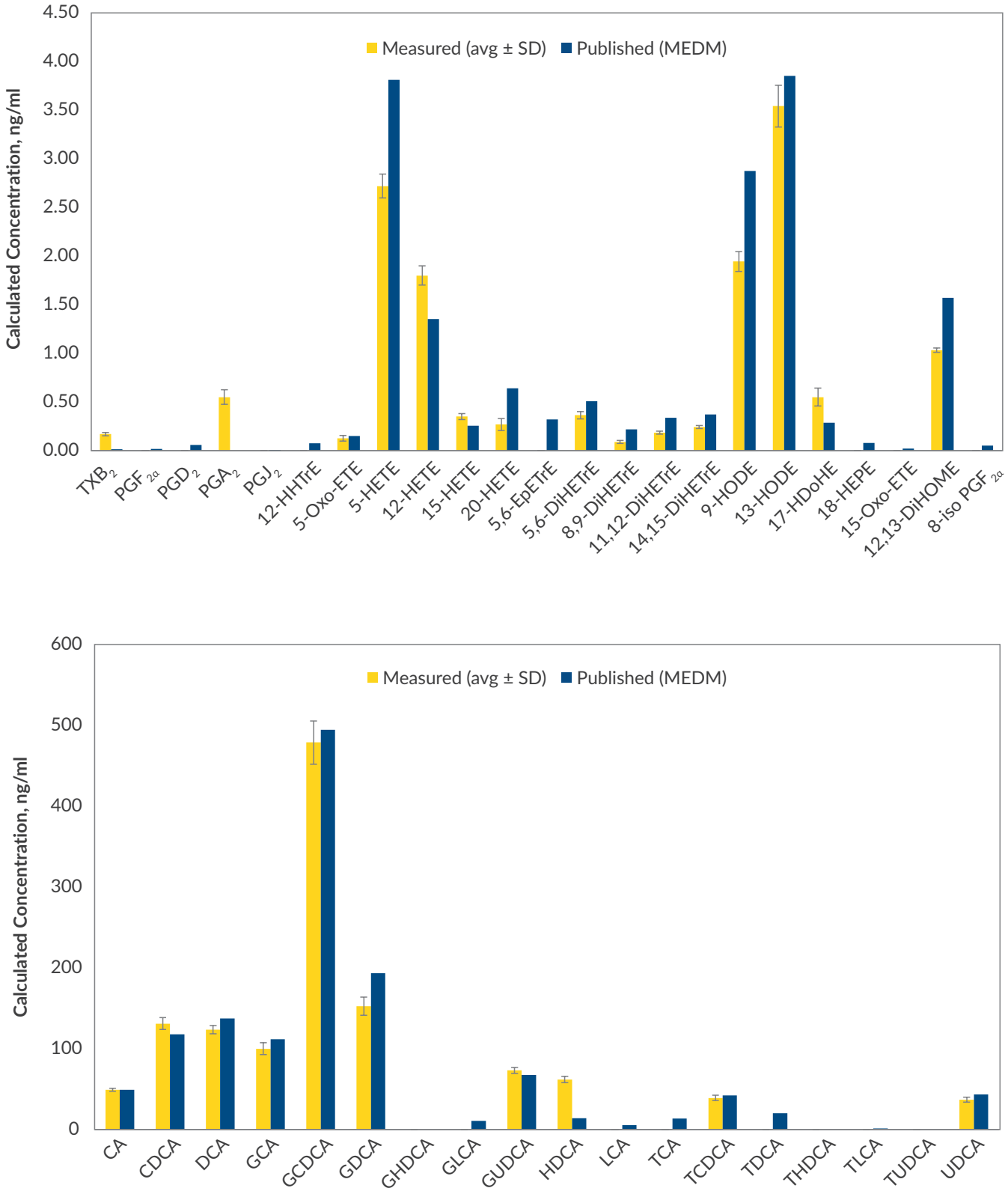

Figure 2. Validation of the methods used for targeted analysis of oxylipins (top) and bile acids (bottom) in NIST reference plasma SRM 1950. The measured data is presented as avg ± SD (n=15; 3 replicate extractions, 5 injections each) and the published data is the consensus MEDM (median of the means).

Table 2. Validation of the method used for untargeted lipidomics analysis in NIST reference plasma SRM 1950. Data shown are the percent of each molecular species in its class, with only the five most abundant lipid molecular species represented for clarity. Values shown are the averages of 12 replicates (3 replicate extractions, 4 injections each).

Molecular Species (Sum Composition)	Published Values (MEDM)	Measured Values (This Study)
CAR(18:1)	N/A	37.9
CAR(18:2)	N/A	18.5
CAR(16:0)	N/A	17.9
CAR(14:1)	N/A	9.4
CAR(18:0)	N/A	6.9
DG(36:3)	15.7	18.6
DG(34:0)	12.2	6.6
DG(36:2)	11.6	13.4
DG(34:1)	11.4	18.8
DG(34:2)	8.2	14.9
TG(52:3)	16.9	15.1
TG(52:4)	8.1	10.6
TG(50:2)	7.9	7.5
TG(52:2)	7.4	14.0
TG(50:1)	6.4	5.3
LPC(16:0)	46.4	43.9
LPC(18:0)	17.2	13.5
LPC(18:2)	14.0	12.6
LPC(18:1)	11.4	10.4
LPC(20:4)	3.8	2.9
PC(34:2)	20.3	34.9
PC(36:4)	12.7	1.6
PC(36:2)	11.8	22.7
PC(34:1)	10.2	2.2
PC(36:3)	8.5	13.9

Molecular Species (Sum Composition)	Published Values (MEDM)	Measured Values (This Study)
LPE(18:2)	23.8	29.4
LPE(18:0)	20.0	21.8
LPE(18:1)	17.5	15.8
LPE(20:4)	13.8	9.8
LPE(16:0)	11.4	13.1
PE(38:4)	15.1	7.5
PE(36:2)	12.5	6.0
PE(O-38:7)	6.5	2.7
PE(38:6)	6.0	7.1
PE(36:4)	5.8	2.0
PI(38:4)	39.5	34.1
PI(36:2)	16.0	23.2
PI(38:3)	7.1	8.7
PI(36:4)	6.2	6.4
PI(34:2)	5.8	6.2
Cer(42:1;O2)	34.9	39.3
Cer(42:2;O2)	15.1	19.0
Cer(41:1;O2)	12.3	11.4
Cer(40:1;O2)	12.0	12.6
Cer(34:1;O2)	5.1	3.4
SM(34:1;O2)	30.0	31.5
SM(42:2;O2)	13.2	14.5
SM(36:1;O2)	6.0	5.7
SM(40:1;O2)	6.0	8.5
SM(42:1;O2)	6.0	5.7
CE(18:2)	55.4	49.6
CE(18:1)	14.7	9.5
CE(20:4)	11.4	25.8
CE(16:0)	6.8	1.2
CE(16:1)	3.3	1.0

The three validated methods were utilized to characterize the MaxSpec® reference plasma. Tables 3 and 4 show the calculated concentrations of bile acids and oxylipins detected in this reference plasma, whereas Figure 3 summarizes the distribution of hundreds of molecular species of lipids in this material.

A list of quantified lipid molecular species in this study is available online.

Table 3. Calculated concentrations of bile acids in MaxSpec® reference plasma. (B)LLOQ, (below) lower limit of quantitation.

Bile Acids	Shorthand	Calibration Standard (Item No.)	LLOQ (ng/ml)	Concentration (ng/ml) Avg ± SD (n=15)
Cholic Acid	CA	31347	3.8	108.6 ± 7.58
Chenodeoxycholic Acid	CDCA	31365	20.8	235.2 ± 15.15
Deoxycholic Acid	DCA	31349	14.1	189.7 ± 7.8
Glycocholic Acid	GCA	31351	8.4	28.3 ± 1.38
Glycochenodeoxycholic Acid	GCDCA	31363	7.7	173.3 ± 7.55
Glycodeoxycholic Acid	GDCA	31599	7.4	58.1 ± 4.52
Glycohyodeoxycholic Acid	GHDCA	31600	14.1	BLLOQ
Glycolithocholic Acid	GLCA	31601	14.9	BLLOQ
Glycoursodeoxycholic Acid	GUDCA	31602	8.1	14.8 ± 1.46
Hyodeoxycholic Acid	HDCA	31606	40.5	40.7 ± 5.31
Lithocholic Acid	LCA	31353	15.3	BLLOQ
Taurocholic Acid	TCA	31374	33.8	BLLOQ
Taurochenodeoxycholic Acid	TCDCA	31361	31.2	BLLOQ
Taurodeoxycholic Acid	TDCA	31603	31.2	BLLOQ
Taurohyodeoxycholic Acid	THDCA	31614	1.9	BLLOQ
Taurolithocholic Acid	TLCA	31604	60.5	BLLOQ
Tauroursodeoxycholic Acid	TUDCA	31605	67	BLLOQ
Ursodeoxycholic Acid	UDCA	31367	14.1	17.6 ± 1.8

Table 4. Calculated concentrations of oxylipins in MaxSpec® reference plasma. (B)LLOQ, (below) lower limit of quantitation; *Surrogate standard.

Oxylipins	Shorthand	Calibration Standard (Item No.)	LLOQ (ng/ml)	Concentration (ng/ml) Avg ± SD (n=15)
Thromboxane B_2	TXB_2	19030	0.04	3.32 ± 0.17
Prostaglandin $F_{2\alpha}$	$PGF_{2\alpha}$	10007211*	0.12	1 ± 0.1
Prostaglandin D_2	PGD_2	10007211*	0.12	0.41 ± 0.04
Prostaglandin A_2	PGA_2	18500*	0.12	24.49 ± 0.97
Prostaglandin J_2	PGJ_2	18500	0.12	3.17 ± 0.23
12-Hydroxy-heptadecatrienoic Acid	12-HHTrE	34590	0.12	1.03 ± 0.08
5-Oxo-eicosatetraenoic Acid	5-Oxo-ETE	34250	0.04	132.49 ± 6.19
5-Hydroxy-eicosatetraenoic Acid	5-HETE	34210	0.01	2738 ± 101.11
12-Hydroxy-eicosatetraenoic Acid	12-HETE	34550	0.04	1477 ± 105.25
15-Hydroxy-eicosatetraenoic Acid	15-HETE	34550*	0.04	1126 ± 59.57
20-Hydroxy-eicosatetraenoic Acid	20-HETE	10007269	0.04	0.71 ± 0.13
5,6-Epoxy-eicosatrienoic Acid	5,6-EpETrE	50211	0.37	BLLOQ
8,9-Epoxy-eicosatrienoic Acid	8,9-EpETrE	50211*	0.37	BLLOQ
11,12-Epoxy-eicosatrienoic Acid	11,12-EpETrE	50511	0.01	BLLOQ
5,6-Dihydroxy-eicosatrienoic Acid	5,6-DiHETrE	51211	0.01	2.44 ± 0.09
8,9-Dihydroxy-eicosatrienoic Acid	8,9-DiHETrE	51211*	0.01	2.88 ± 0.1
11,12-Dihydroxy-eicosatrienoic Acid	11,12-DiHETrE	51511	0.01	1.01 ± 0.05
14,15-Dihydroxy-eicosatrienoic Acid	14,15-DiHETrE	51511*	0.01	2.12 ± 0.11
9-Hydroxy-octadecadienoic Acid	9-HODE	38400	0.01	2743 ± 139.27
13-Hydroxy-octadecadienoic Acid	13-HODE	38400*	0.01	3213 ± 118.34
17-Hydroxy-docosahexaenoic Acid	17-HDoHE	33650	0.12	520 ± 31.56
18-Hydroxy-eicosapentaenoic Acid	18-HEPE	32840	0.04	22.46 ± 1.07
15-Oxo-eicosatetraenoic Acid	15-Oxo-ETE	34550*	0.04	652.40 ± 38.71
12,13-Dihydroxy-octadecamonoenoic Acid	12,13-DiHOME	10009832	0.01	107.11 ± 3.24
9,10-Dihydroxy-octadecamonoenoic Acid	9,10-DiHOME	10009832*	0.01	137.85 ± 4.36
8-Iso-prostaglandin F2α	8-iso-$PGF_{2\alpha}$	18500*	0.12	2.5 ± 0.46

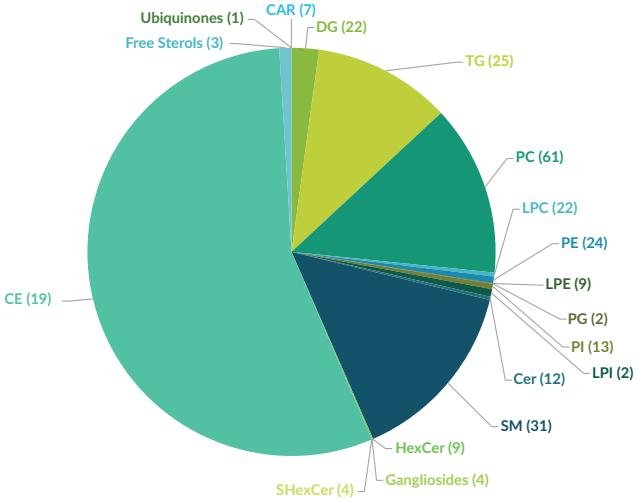

Figure 3. Distribution of lipid molecular species by their measured abundance in MaxSpec® reference plasma.

As additional characterization of both the MaxSpec® plasma and the untargeted lipidomics method, we explored the effect that decreasing volumes of plasma extracted would have in the number of molecular species identified. As expected, the total number of detectable molecular species is higher with higher volumes extracted, as shown in Table 5. However, the increase of identifications is relatively modest above 2 µl. Of note, the untargeted analysis utilized allows for the identification and quantitation of over a hundred lipid molecular species from sub-microliter volumes of human plasma.

Table 5. Effect of volume extracted on the number of curated molecular species detectable in the MaxSpec® reference plasma.

Lipid Class	Volume of Plasma Extracted (µl)				
	60	20	6.7	2.2	0.74
CAR	6	6	2	0	0
MG	2	2	2	2	0
DG	28	26	26	27	14
TG	82	82	82	77	36
PA	3	3	3	1	0
PC	63	61	56	49	27
PE	38	38	36	26	11
PG	6	5	5	3	1
PI	21	21	20	18	9
PS	3	3	2	0	0
Cer	22	22	21	18	4
S1P	1	0	0	0	0
SM	23	23	23	20	8
Gangliosides	6	6	6	6	0
SHexCer	3	3	3	2	0
HexCer	10	9	8	8	3
Sterols	21	19	15	15	6
CoQ10	1	1	1	0	0
Total	**339**	**330**	**311**	**272**	**119**

Additional characterization of this reference plasma preparation is ongoing to increase the coverage of quantified lipids and to evaluate the stability of analytes, which will progressively increase the usefulness of this material as a reference control.

References

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