Supplement material and methods

Bioanalytical Quantification of Cyclophosphamide

Chromatographic separation was performed using an Agilent 1260 HPLC system coupled to an API 3200 triple quadrupole mass spectrometer on a Thermo Scientific Hypersil BDS C18 column (2.1 × 100 mm, 3.0 μm). The mobile phase consisted of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). Gradient elution was performed over a 7-minute run time at a flow rate of 0.2 mL/min. The detailed LC-MS/MS gradient program is provided in Supplementary Table 1.

Table 1. LC-MS/MS gradient program for cyclophosphamide analysis.

Step	Time (min)	Flow rate (μL/min)	Mobile Phase A (%)	Mobile Phase B (%)
0	0.00	200.0	5.0	95.0
1	1.00	200.0	5.0	95.0
2	2.00	200.0	5.0	95.0
3	3.00	200.0	95.0	5.0
4	4.00	200.0	95.0	5.0
5	5.00	200.0	95.0	5.0
6	6.00	200.0	5.0	95.0
7	7.00	200.0	5.0	95.0

Sample Preparation

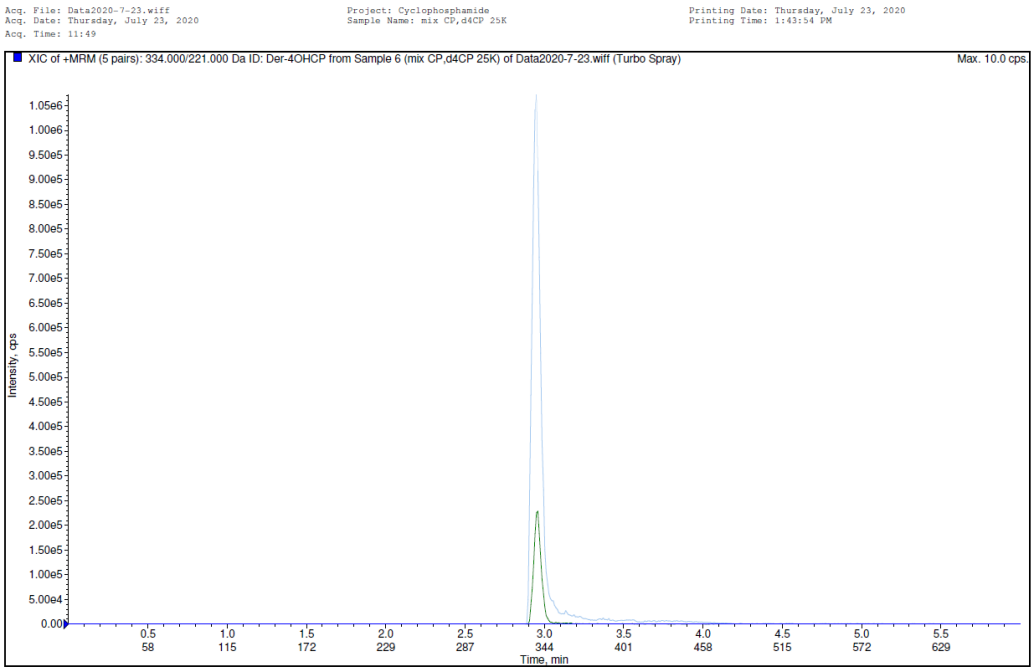
Plasma samples were prepared using liquid–liquid extraction. The LC–MS/MS assay was validated over a calibration range of 100–20,000 ng/mL using calibrators and quality-control samples prepared within this range. For patient sample analysis, all plasma samples were routinely diluted four-fold before extraction by mixing 25 μL of patient plasma with 75 μL of blank plasma. This dilution was applied to patient samples to bring cyclophosphamide concentrations within the validated calibration range.

Following dilution, 10 μL of cyclophosphamide-d4 internal standard (4,000 ng/mL) was added. Samples were extracted with 250 μL of ethyl acetate, vortex-mixed, and centrifuged at 14,400 rpm for 5 minutes. The organic layer was transferred to a clean tube and evaporated to dryness under vacuum at 50°C for 20 minutes. The residue was reconstituted in 100 μL of a solution containing 5% mobile phase A and 95% mobile phase B, and 1 μL was injected into the LC–MS/MS system. Following analysis, the measured cyclophosphamide concentration was multiplied by a dilution factor of 4 to obtain the concentration in the original plasma sample. Dilution integrity of the four-fold pre-extraction dilution was not formally evaluated during assay validation.

LC-MS/MS system

Detection was performed using positive electrospray ionization with multiple reaction monitoring (MRM). Cyclophosphamide and cyclophosphamide-d_4 were monitored using the transitions m/z 261→140 and m/z 265→140, respectively. The retention time of both cyclophosphamide and the internal standard was approximately 2.95 minutes. Representative chromatograms are shown in Supplementary Figure 1. Instrument parameters for ion monitoring, including the declustering potential (DP), entrance potential (EP), collision energy (CE), collision cell entrance potential (CEP), and collision cell exit potential (CXP), are provided in Supplementary Table 2.

Figure 1. Representative LC-MS/MS chromatograms showing cyclophosphamide and d4-cyclophosphamide with a retention time of 2.95 min.



The separation of cyclophosphamide and the d4-CP internal standard was successfully accomplished at a flow rate of 0.2 mL/min. Both cyclophosphamide and d4-CP had a retention time of 2.95 minutes. The injection volume of 1 µl was injected every 7 min.

Table 2. Ion source and analyte-dependent mass spectrometric parameters for LC-MS/MS analysis of cyclophosphamide.

Part A. MRM transitions and compound-specific parameters

Analyte	Q1 (m/z)	Q3 (m/z)	Dwell time (ms)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Cyclophosphamide	261	140	200	100.22	3.88	16.72	27.5	2.48
d4-Cyclophosphamide (IS)	265	140	200	57.01	6	22.17	31.94	2.2

Part B. Ion source parameters

Parameter	Value
Curtain gas (CUR)	20
Collision gas (CAD)	5
Ion source gas 1 (GS1)	50
Ion source gas 2 (GS2)	50
Ion spray voltage (IS)	5500 V
Source temperature (TEM)	500 °C
Interface heater	ON

Abbreviations: Q1, precursor ion; Q3, product ion; DP, declustering potential; EP, entrance potential; CEP, collision cell entrance potential; CE, collision energy; CXP, collision cell exit potential; CUR, curtain gas; CAD, collision gas; GS1, ion source gas 1; GS2, ion source gas 2; IS, Ion Spray voltage; TEM, source temperature; IS (internal standard), d4-cyclophosphamide.

Analyst® 1.5.2 Software (AB Sciex, Framingham, MA) was used for integration of peak areas and evaluation of data. The method of weighted (1/x) quadratic regression was used for determination of concentrations.

Bioanalytical Method Validation

The LC-MS/MS method was validated according to the United States Food and Drug Administration (FDA) Guidance for Industry: Bioanalytical Method Validation (2018). The validated LC-MS/MS assay demonstrated excellent linearity over the calibration range of 100–20,000 ng/mL, with a correlation coefficient (*R*) of 0.9998, and exhibited acceptable accuracy, precision, extraction recovery, matrix effects, and stability in accordance with FDA bioanalytical validation criteria. Calibration curve performance is summarized in Supplementary Table 3.

Table 3. Linearity and reproducibility of the cyclophosphamide calibration curve.

Nominal concentration (ng/mL)	Day					Mean	SD	%CV
	1	2	3	4	5			
100	100	103	100	99	109	102.2	4.09	4.00
500	463	535	491	499	447	487	34.06	6.99
1000	905	949	984	1010	902	950	47.66	5.02
2000	2020	1870	1970	1970	1890	1944	62.29	3.20
4000	4090	3830	3990	3920	3930	3952	96.02	2.43
6000	6090	5900	6090	6090	6050	6044	82.34	1.36
10000	9820	10400	9940	10100	10300	10112	241.50	2.39
15000	15200	15400	15100	14800	15200	15140	219.09	1.45
20000	19900	19600	20000	20100	19700	19860	207.36	1.04
Regression equation	y = 1.09e-009 x^2 + 0.00034 x + 0.00784	y = 1.35e-009 x^2 + 0.000319 x + -0.0048	y = 1.83e-009 x^2 + 0.000476 x + -0.00621	y = 3.42e-009 x^2 + 0.000346 x + -0.00164	y = 2.89e-009 x^2 + 0.000303 x + -0.00304			
R^2	0.9998	0.9998	1	0.9999	0.9996	0.99982		

Abbreviations: SD, standard deviation; CV, coefficient of variation; R^2, coefficient of determination.

Calibration curves were prepared over the concentration range of 100–20,000 ng/mL.

The lower limit of quantification (LLOQ) was 100 ng/mL. Intra-day accuracy ranged from 94.71% to 101.73%, whereas inter-day accuracy ranged from 94.84% to 101.47%. Intra- and inter-day precision, expressed as the coefficient of variation (% CV), ranged from 0.90% to 2.45% across all quality-control concentrations. Accuracy and precision data are summarized in Supplementary Table 4.

Table 4. Accuracy and precision of the LC-MS/MS method for the determination of cyclophosphamide.

Analyte	Nominal concentration (ng/mL)	Intra-day (n = 6)			Inter-day (n = 18)		
		Calculated concentration (ng/mL) Mean ± SD	Accuracy (%)	Precision (% CV)	Calculated concentration (ng/mL) Mean ± SD	Accuracy (%)	Precision (% CV)
Cyclophosphamide	100	101.68 ± 1.52	101.73	1.34	101.47 ± 2.48	101.47	2.45
	300	296.6 ± 2.88	98.78	0.90	299.5 ± 5.43	99.83	1.80
	8000	7548 ± 51.67	94.71	1.11	7587.22 ± 117.90	94.84	1.55
	16000	15900 ± 282.84	99.38	1.59	15677.78 ± 305.78	97.99	1.95

Accuracy is expressed as the percentage of the nominal concentration, and precision is expressed as the coefficient of variation (% CV).

Mean extraction recovery ranged from 90.6% to 96.1%, and matrix effects ranged from 98.1% to 101.4%, indicating efficient analyte recovery with minimal matrix interference.

Cyclophosphamide stability was evaluated under autosampler, bench-top (room temperature), freeze–thaw, and long-term storage conditions according to the validated bioanalytical protocol. Cyclophosphamide remained stable under all evaluated conditions, with measured concentrations ranging from 89.1% to 104.7% of nominal values. Long-term stability was demonstrated for up to 3 years at −80°C, exceeding the storage duration of the study samples. Following collection and processing, patient plasma samples were stored at −80°C until LC–MS/MS analysis and were analyzed in batches within 1 year of collection. Stability data are presented in Supplementary Table 5.

Table 5. Stability of cyclophosphamide under different storage conditions.

Stoarage condition	Peak Area		Accuracy (%)
	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	
Autosampler 24 hrs	292.6	278.00	95.01
	16240	16080.00	99.01
RT 24 hrs	292.6	303.40	103.69
	16240	16320.00	100.49
7 DAY (4°C)	292.6	292.80	100.07
	16240	16580.00	102.09
7 DAY (-20°C)	292.6	280.60	95.90
	16240	16380.00	100.86
7 DAY (-80°C)	292.6	293.60	100.34
	16240	16520.00	101.72
1 Month (4°C)	292.6	297.80	101.78
	16240	15720.00	96.80
1 Month (-20°C)	292.6	302.60	103.42
	16240	15860.00	97.66
1 Month (-80°C)	292.6	306.40	104.72
	16240	15380.00	94.70
2 Month (4°C)	292.6	288.60	98.63
	16240	15900.00	97.91
2 Month (-20°C)	292.6	278.40	95.15
	16240	16480.00	101.48
2 Month (-80°C)	292.6	299.60	102.39
	16240	15780.00	97.17
3 Month (4°C)	292.6	270.8	92.55
	16240	15260	93.97
3 Month (-20°C)	292.6	260.8	89.13
	16240	14960	92.12
3 Month (-80°C)	292.6	276.2	94.40
	16240	15000	92.36
1 Year (-20°C)	292.6	274	93.64
	16240	15540	95.69
1 Year(-80°C)	292.6	281.8	96.31
	16240	15980	98.40
2 Year (-20°C)	292.6	287.80	98.36
	16240	16540.00	101.85
2 Year (-80°C)	292.6	286.60	97.95
	16240	16400.00	100.99
3 Year (-80°C)	292.6	281.60	96.24
	16240	16140.00	99.38
3 Year (-80°C)	292.6	282.60	96.58
	16240	15400.00	94.83
FT (-20°C)	292.6	297.00	101.50
	16240	16100.00	99.14
FT (-80°C)	292.6	297.60	101.71
	16240	15940.00	98.15

Abbreviations: RT, room temperature; FT, freeze–thaw.

Pharmacogenetic Analysis

Polymerase chain reaction (PCR) amplification was performed using a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). SNP genotyping of *GSTP1*, *ALDH1A1*, and *ALDH3A1* was performed using gene-specific PCR followed by Sanger sequencing. *GSTT1* and *GSTM1* genotypes were determined in all 30 patients using multiplex PCR followed by agarose gel electrophoresis, with β-globin included as an internal amplification control. The expected PCR product sizes were 459 bp for *GSTT1*, 214 bp for *GSTM1*, and 296 bp for β-globin. The presence of a gene-specific band at the expected size was interpreted as the corresponding gene-present genotype, whereas absence of the gene-specific band in the presence of successful β-globin amplification was interpreted as the corresponding null genotype. For additional confirmation of the multiplex PCR results, five selected patients with a *GSTT1*-present genotype underwent Sanger sequencing.

Separate optimized thermal cycling protocols were used for Sanger sequencing PCR (34 cycles) and multiplex PCR (30 cycles). Multiplex PCR was performed using an annealing temperature of 58 °C, and amplification products were separated on a 2% agarose gel at 110 V for 36 min. Primer sequences and detailed PCR cycling conditions are provided in Supplementary Tables 6 and 7, respectively. Representative Sanger sequencing chromatograms of pharmacogenetic variants are provided in Supplementary Figure S1, and representative multiplex PCR gel electrophoresis for *GSTT1* and *GSTM1* genotyping is shown in Supplementary Figure S2.

Table 6. Primer sequences used for PCR amplification of pharmacogenetic variants.

Gene	Sequence	Size	Tm	Product
GSTP1				
rs1695 c.313A>G	5'- TCTCTCATCCTTCCACGCACATCCTCTT -3'	28	62.5	735
	5'- TCTCCTTACCCACCCGACACAAAT -3'	24	60	
GSTT1				
null gene	5'- TTC CTT ACT GGT CCT CAC ATC TC -3'	23	55.8	459
	5'- TCA CCG GAT CAT GGC CAG CA -3'	20	61.7	
GSTM1				
null gene	5'- ATG GTT TGC AGG AAA CAA GG -3'	20	53.8	214
	5'- AAA GCG GGA GAT GAA GTC CT -3'	20	56.1	
ALDH3A1				
rs2228100 c.985C>G	5'- AAT TCT GGC AGG GCA TCA -3'	18	54.5	641
	5'- GCA AGG ATC GCT CAT TCA GT -3'	20	55.3	
ALDH1A1				
rs6151031	5'- GTTTGGAAGCCCTGCTTAAAT -3'	21	59	580
	5'- CAAAGCCGAAACCTGTGATAAG -3'	22	59.8	

Abbreviations: Tm, melting temperature; bp, base pairs

Table 7. PCR cycling conditions for primer optimization using a Bio-Rad T100 Thermal Cycler.

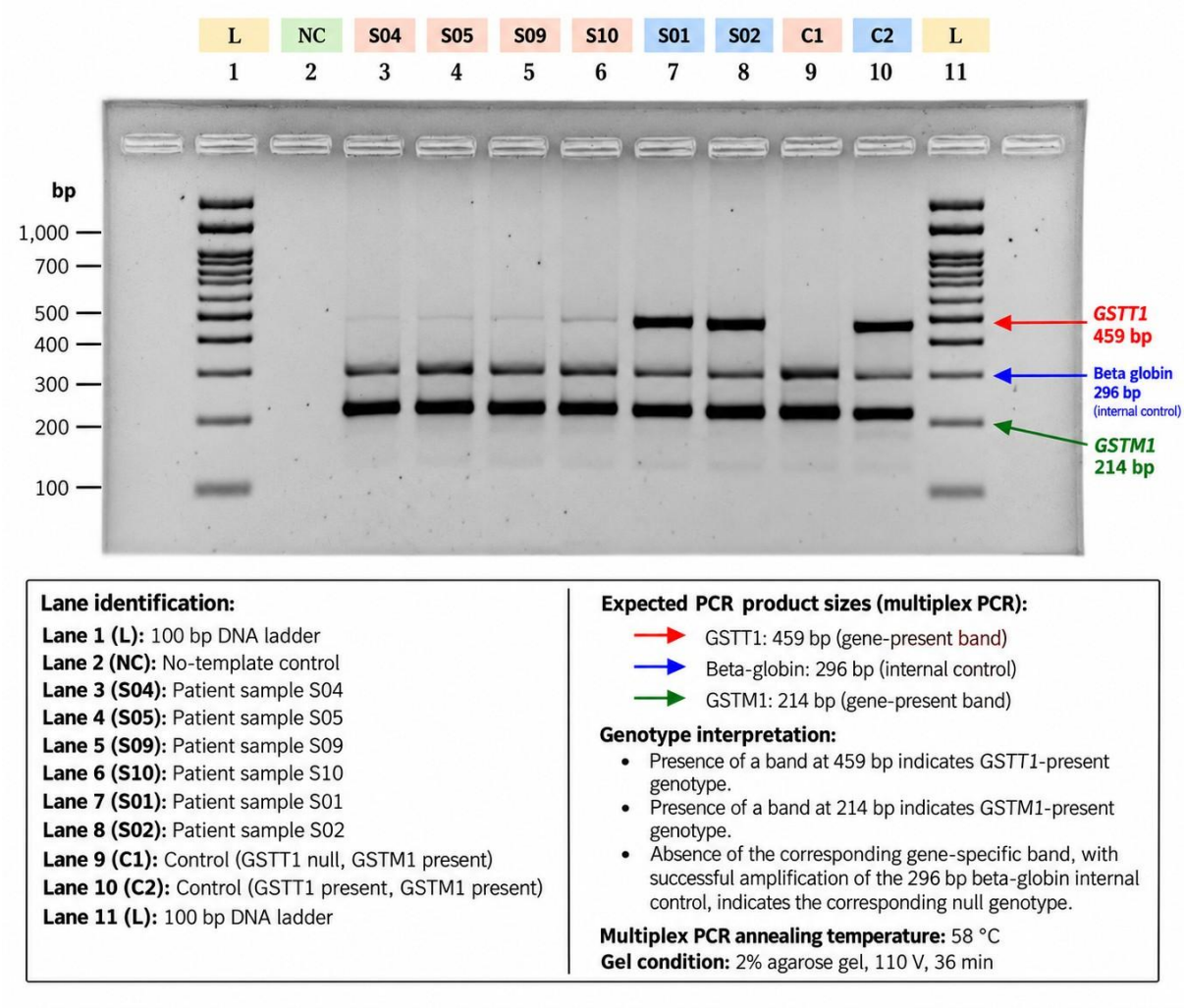
Part A. Thermal cycling conditions for *CYP2B6*, *GSTP1*, *ALDH1A1*, and *ALDH3A1*2*.

Cycle step	Temperature (°C)	Time	Cycles
Initial denaturation	98	30 s	1
Denaturation	98	10 s	34
Annealing	65	30 s	
Extension	72	30 s	
Final extension	72	5 min	1
Hold	4	Hold	—

Part B. Thermal cycling conditions for *GSTT1* and *GSTM1* (multiplex PCR).

Cycle step	Temperature (°C)	Time	Cycles
Initial denaturation	98	3 min	1
Denaturation	95	30 s	30
Annealing	56.8	30 s	
Extension	72	45 s	
Final extension	72	10 min	1
Hold	4	Hold	—

Figure 2. Multiplex PCR analysis of GSTT1 and GSTM1 gene-present and null genotypes.



Representative multiplex PCR gel electrophoresis for *GSTT1* and *GSTM1* genotyping. PCR products were separated by agarose gel electrophoresis, and genotypes were determined according to the presence or absence of gene-specific amplification products. The expected product sizes were 459 bp for *GSTT1* and 214 bp for *GSTM1*, with a 296-bp β-globin product serving as the internal control. The presence of a band at the expected size indicated the corresponding gene-present genotype, whereas absence of the gene-specific band, in the presence of successful β-globin amplification, was interpreted as the corresponding null genotype. Selected *GSTT1*-present samples were subsequently confirmed by Sanger sequencing.