Electronic Supplementary Material (ESI) for Analyst.

A high-specificity immunoassay for the therapeutic drug monitoring of cyclophosphamide

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SUPPORTING INFORMATION

S1. Synthesis.

S1.1 Reagents and instruments.

The chemicals used in the synthesis of the haptens were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA) or Sigma Chemical Co. (St. Louis, MO, USA) Cyclophosphamide monohydrate was purchased form Alfa Aesar (Haverhill, MA, USA) and Carboxycyclophosphamide from Toronto research chemicals (North York, Canada). Thin-layer chromatography (TLC) was performed on 0.25 mm silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany). ^{1}H and ^{13}C NMR spectra were obtained with a Varian Mercury-400 spectrometer (400 MHz ^{1}H and 101 MHz for ^{13}C). Liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS) was performed in a Waters (Milford, MA, USA) model composed by an Acquity UPLC system directly interfaced to a Micromass LCT Premier XE MS system equipped with an ESI LockSpray source for monitoring positive ions. Data were processed with MassLynx (V4.1) software (Waters).

S1.2 Synthetic characterization.

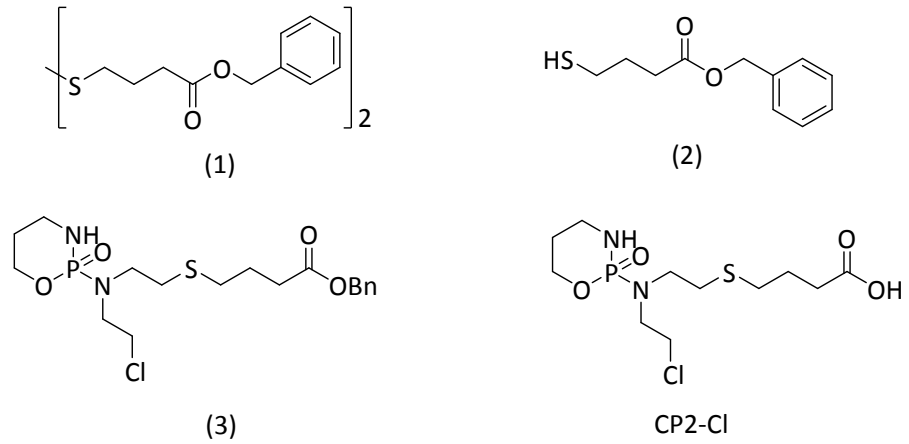

Figure S1.1. *Structure of synthesized compounds.*

Dibenzyl 4,4'-disulfanediyldibutyrate (1). **^{1}H NMR** (400 MHz, Chloroform-*d*): δ 7.36 (d, *J* = 3.7 Hz, 4H), 5.12 (s, 2H), 2.69 (t, *J* = 7.1 Hz, 2H), 2.48 (t, *J* = 7.3 Hz, 2H), 2.04 (q, *J* = 7.2 Hz, 2H). **^{13}C NMR** (101 MHz, Methanol-d_4): δ 174.36, 137.60, 129.53, 129.22, 129.19, 67.26, 38.46, 33.41, 25.37. **HRMS** (+ESI): *m/z* calculated for $C_{22}H_{27}O_4S_2$ $[M + H]^+$ 419.1351, found 419.1338 (-3.1ppm).

Benzyl 4-thiobutyrate (2). **^{1}H NMR** (400 MHz, Methanol-d_4): δ 7.44 – 7.18 (m, 4H), 5.10 (d, *J* = 3.7 Hz, 2H), 2.61 – 2.33 (m, 4H), 1.97 – 1.73 (m, 2H). **^{13}C NMR** (101 MHz, Methanol-d_4): δ 174.45, 137.56, 129.51, 129.15, 67.19, 33.44, 30.23, 24.30.

Benzyl 4-((2-((2-chloroethyl)(2-oxido-1,3,2-oxazaphosphinan-2-yl)amino)ethyl)thio) butanoate (3). **^{1}H NMR** (400 MHz, Methanol-d_4): δ 7.47 – 7.27 (m, 5H), 5.16 (s, 2H), 4.44 – 4.22 (m, 2H), 3.66 (t, J = 7.3 Hz, 2H), 3.49 – 3.15 (m, 8H), 2.75 – 2.65 (m, 2H), 2.61 (t, J = 7.2 Hz, 2H), 2.53 (t, J = 7.2 Hz, 2H), 2.00 – 1.72 (m, 3H). **^{13}C NMR** (101 MHz, Methanol-d_4): δ 174.50 , 137.61 , 129.53 , 129.17 , 69.30 (d, J = 6.9 Hz), 67.21 , 42.98 (d, J = 1.6 Hz), 42.07 (d, J = 2.7 Hz), 33.69 , 31.83 , 31.34 (d, J = 1.4 Hz), 26.85 (d, J = 6.3 Hz), 25.91. **HRMS** (+ESI): *m/z* calculated for $C_{18}H_{29}N_2O_4PSCl$ $[M + H]^+$ 435.1247, found 435.1228 (-10.6 ppm); (-ESI): *m/z* calculated for $C_{19}H_{29}ClN_2O_6PS$ $[(M + HCOOH)-H]^-$ 479.1172, found 479.1140 (-6.7 ppm).

4-((2-((2-chloroethyl)(2-oxido-1,3,2-oxazaphosphinan-2-yl)amino)ethyl)thio)butanoic acid (CP2-Cl). **^{1}H NMR** (400 MHz, Methanol-d_4): δ 4.43 – 4.22 (m, 2H), 3.70 – 3.58 (m, 2H), 3.46 – 3.15 (m, 2H), 2.76 – 2.65 (m, 2H), 2.60 (t, *J* = 7.2 Hz, 2H), 2.43 (t, *J* = 7.2 Hz, 2H),

1.96 – 1.74 (m, 4H). **^{13}C NMR** (101 MHz, Methanol-d_4): δ 176.79, 69.37 (d, J = 6.9 Hz), 49.36 (d, J = 5.0 Hz), 48.08 (d, J = 4.1 Hz), 42.97 (d, J = 1.7 Hz), 42.10 (d, J = 2.7 Hz), 33.52 , 31.92 , 31.36 (d, J = 1.6 Hz), 26.87 (d, J = 6.4 Hz), 25.99. **HRMS** (+ESI): *m/z* calculated for $C_{11}H_{23}N_2O_4PSCl$ $[M + H]^+$ 345.0805, found 345.0812 (2.0 ppm); (-ESI): *m/z* calculated for $C_{11}H_{21}N_2O_4PSCl$ $[M - H]^-$ 343.0648, found 343.0648 (0 ppm).

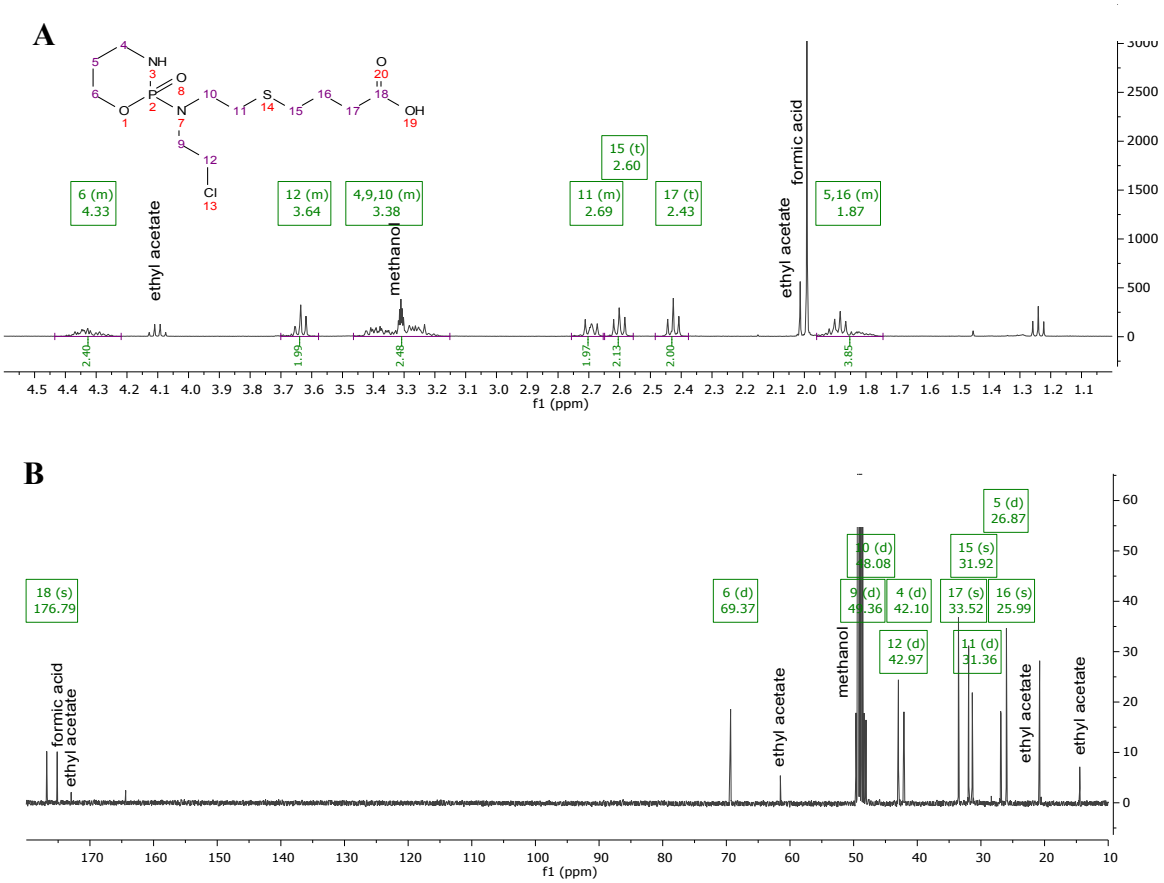

Figure S1.2. *(A) ^{1}H NMR spectra of CP-2Cl. (B) ^{13}C NMR spectra of CP-2Cl. Assigned peaks were confirmed by HSQC and HMBC.*

S2. Immunochemistry.

S2.1 Instruments and General Methods.

The chemical reagents used were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA) and from Sigma Chemical Co. (St. Louis, MO, USA). The matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS) was a Bruker

autoflex III Smartbeam spectrometer (Billerica, Massachusetts). The pH and the conductivity of all buffers and solutions were measured with a pH-meter pH 540 GLP and a conductimeter LF 340, respectively (WTW, Weilheim, Germany). Antigens were purified using Cellusep dialysis fragments with a nominal MWCO of 12000-14000 versus 5 L of PBS for 48 h. Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark). Dilution plates were purchased from Nirco (Barberà del Vallés, Spain). Washing steps were performed on a Biotek ELx465 (Biotek Inc.). A Heidolph Titramax 1000 vibrating platform shaker (Brinkmann Instruments, Westbury, NY, USA) was used to shake the microplates at 600 rpm. Absorbances were read on a SpectramaxPlus (Molecular Devices, Sunnyvale, CA, USA). The competitive curves were analyzed with a four-parameter logistic equation using the software GraphPad Prism 5.03 (GraphPad Software Inc., San Diego, CA, USA). Blank serum was purchased from Merck (Ref. S1-100ML #2855937, Darmstadt, Germany).

S2.2 Buffers.

Unless otherwise indicated, phosphate buffer saline (PBS) is 0.01 M phosphate buffer in a 0.8% saline solution, pH 7.5. Coating buffer is a 0.05 M carbonate-bicarbonate buffer, pH 9.6. PBST is PBS with 0.05% Tween 20, pH 7.5. Citrate buffer is 0.04 M sodium citrate, pH 5.5. The substrate solution contains 0.01% 3,3',5,5'-tetramethylbenzidine (TMB) and 0.004% H_2O_2 in citrate buffer. Borate buffer is 0.2 M boric acid/sodium borate, pH 8.7.

S2.3 Hapten densities of the bioconjugates.

Table S2.1 Hapten densities of the immunogen and the competitors[a]

Immunoreagents	δ-hapten[b]
CP2-Cl-HCH	15[c]
CP2-Cl-BSA	4
CXCP-BSA	7
CP2-Cl -CONA	15
CXCP-CONA	10

[a]Analyses were performed by MALDI-TOF-MS. OVA and AD conjugates couldn't be analyzed. [b]Mols of hapten per mol of protein. [c]The immunogen CP2-Cl-HCH characterized using BSA as a protein model.

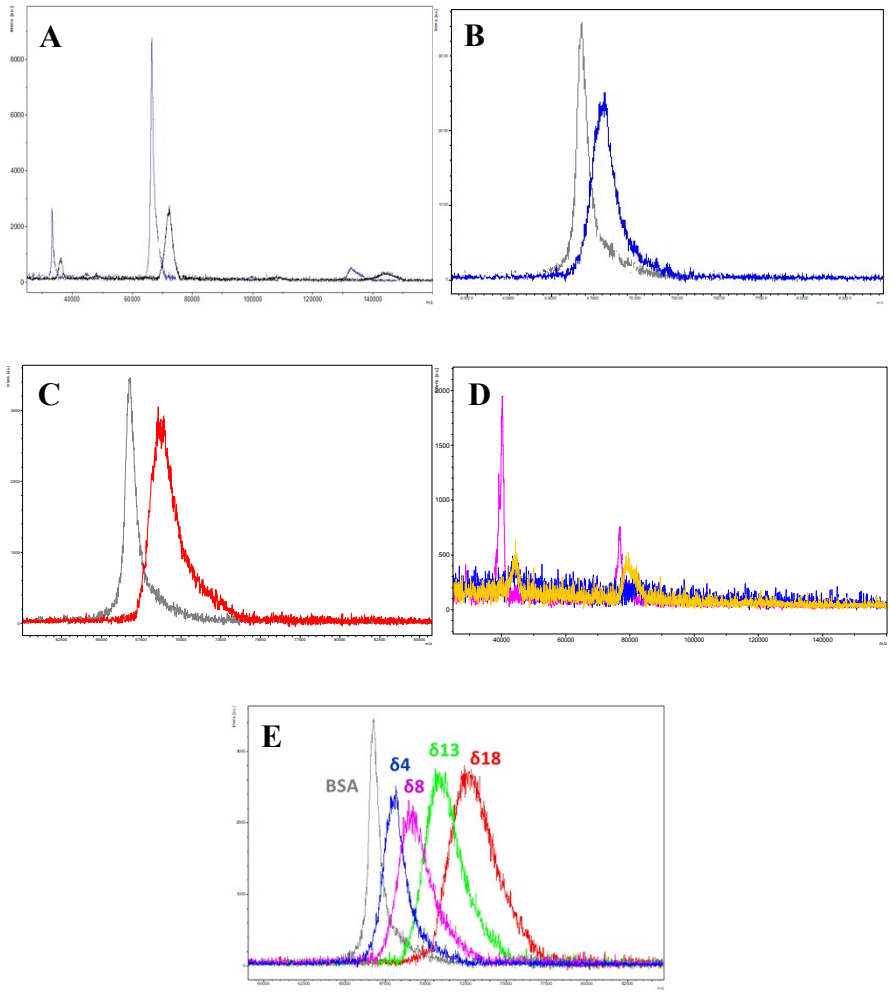

Figure S2.1. *MALDI-TOF-MS spectra of (A) CP2-Cl-HCH protein model CP2-Cl-BSA in black and BSA reference in blue. (B) CP2-Cl-BSA in blue and BSA reference in grey. (C) CXCP-BSA in red and BSA reference in grey. (D) CP2-Cl-CONA in blue and CXCP-CONA in yellow, CONA reference is pink. (E) Different hapten densities obtained with CP2-Cl-BSA.*

S2.4 ELISA optimization.

Standard calibration curves were carried out at different conditions, varying only one parameter individually.

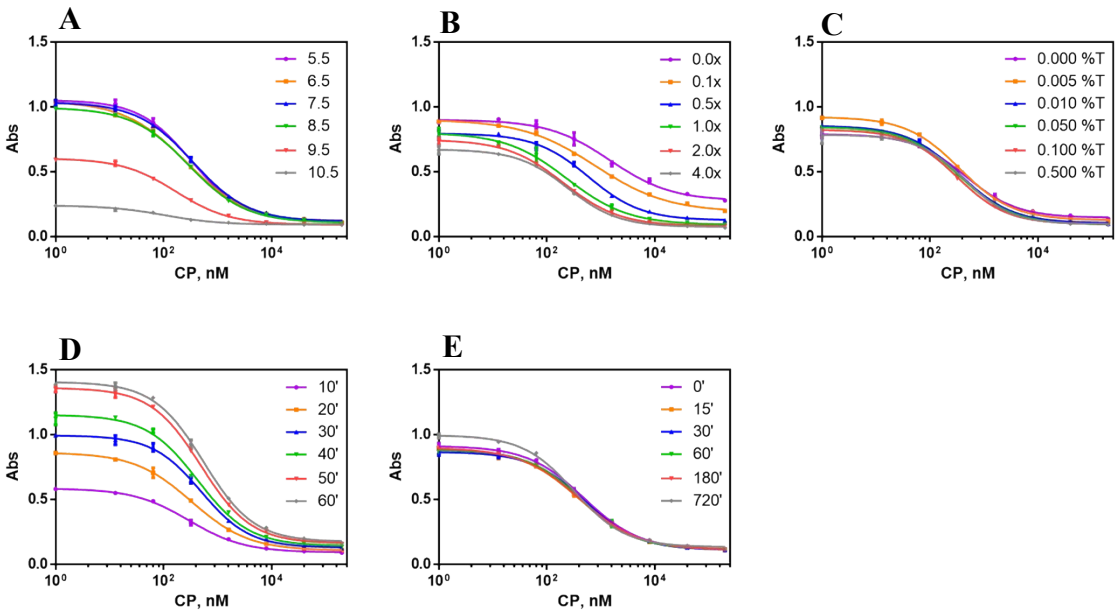

Figure S2.2. *Effect of different physico-chemical parameters on the As343/CXCP-BSA immunoassay. (A) effect of pH; (B) effect of ionic strength (conductivity); (C) effect of Tween 20; (D) effect of the competition time; (E) effect of the incubation time. At least two-well replicates were employed for each assay.*