Prednisolone Assay Description

Two-hundred µL of serum was combined with 600 µL acetonitrile (Fisher Scientific reagent grade, Waltham MA), mixed by vortexing, and centrifuged to remove insoluble particulates. The mixture was passed through a 0.2 µm Pall Bio-inert filter (Pall Corporation, VWR International, Radnor PA) and 250 µL was transferred to an autosampler vial for chromatography. This preparation was used for determination of total serum prednisolone concentrations, and additional descriptions of the assay can be found elsewhere.[1]

Unbound or non-protein bound prednisolone was extracted by adaptation of the method of Ionita and Akhlaghi.[2] In brief, 300 µL of serum were transferred into individual Microcon centrifugal filter devices with Ultracel YM-10 10,000 Da molecular weight cutoff filters (Millipore, Bedford MA). The filters were capped and equilibrated for 60 minutes at 37^{o}C, then centrifuged at 10000 g for 30 minutes in a benchtop microcentrifuge. One hundred µL of ultrafiltrate was combined with 10 µL of internal standard solution comprising 50% acetonitrile and 50 ng/mL d7-prednisone (Toronto Research Chemicals, LGC Standards, Toronto ON). An in-line switching valve on the LC-MS/MS instrument enabled trapping of unbound prednisolone and its internal standard on a Strata-X 25 µm extraction cartridge with dimensions 20 x 2.0 mm (Phenomenex, Torrance CA) with washing and backflushing as previously described.[2]

Analyte separation was achieved using a Shimadzu LC30AD ultrahigh-performance liquid chromatography (UHPLC) system (Shimadzu, Kyoto Japan) followed immediately by a Phenomenex (Torrance, CA) Kinetex Biphenyl 100 angstrom column (30 × 2.1 mm i.d., 2.6 µm). Identification and quantitation were accomplished using an ABSciex 6500+ triple quadrupole mass spectrometer (ABSciex, Framingham MA) with an electrospray ionization source in the negative ionization mode. Chromatographic separation involved a gradient between

mobile phase A (0.1% formic acid [Merck, Millipore Corporation, Bedford MA] in Milli-Q Ultrapure deionized water [Millipore]) and mobile phase B (0.1% formic acid in acetonitrile) which proceeded as 70% A/30% B from 0-1 min, 100% B from 1.0-7.0 min, then 70% A/30% B from 7.0-7.5 min and 70% A/30% B to 8.0 min. A formate adduct of prednisolone and the d7-internal standard generated by this procedure were used to establish MRM settings (see Table S1), which included: 1) prednisolone *m/z* 405→329 quantifier, 405→295 qualifier-1 and 405→45 qualifier-2; and 2) prednisone-d7 *m/z* 410→341 quantifier and 410→110 qualifier.

References:

1. Heine LK, Benninghoff AD, Ross EA, et al. Comparative effects of human-equivalent low, moderate, and high dose oral prednisone intake on autoimmunity and glucocorticoid-related toxicity in a murine model of environmental-triggered lupus. *Front Immunol*. 2022;13:972108.
2. Ionita IA, Akhlaghi F. Quantification of unbound prednisolone, prednisone, cortisol and cortisone in human plasma by ultrafiltration and direct injection into liquid chromatrography tandem mass spectrometry. *Ann Clin Biochem*. 2010;47(Pt 4):350-357.

Table S1. MRM acquisition parameters for prednisone, prednisolone and the prednisone-d7 internal standard for the AB Sciex UPLC/MS/MS system.

Q1 Mass (Da)	Q2 Mass (Da)	Dwell Time (msec)	Target compound	MRM type	DP (volts)	EP (volts)	CE (volts)	CXP (volts)
403.017	327	150	Prednisone 1	Quant	-20	-10	-22	-21
403.017	357.1	150	Prednisone 2	Qual	-20	-10	-14	-27
403.017	299.1	150	Prednisone 3	Qual	-20	-10	-26	-17
405.025	329.1	150	Prednisolone 1	Quant	-15	-10	-22	-21
405.025	295	150	Prednisolone 2	Qual	-15	-10	-44	-23
405.025	280	150	Prednisolone 3	Qual	-15	-10	-46	-23
426.1	109.9	150	IS Prednisone d7 1	IS Quant	-25	-10	-24	-15
426.1	341.2	150	IS Prednisone d7 2	IS Qual	-25	-10	-24	-15