Prednisolone and prednisone detection in urine samples: a GC-C-IRMS method to discriminate their exogenous or endogenous origin

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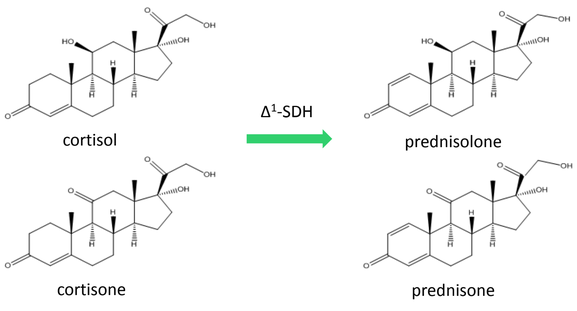
1 Introduction

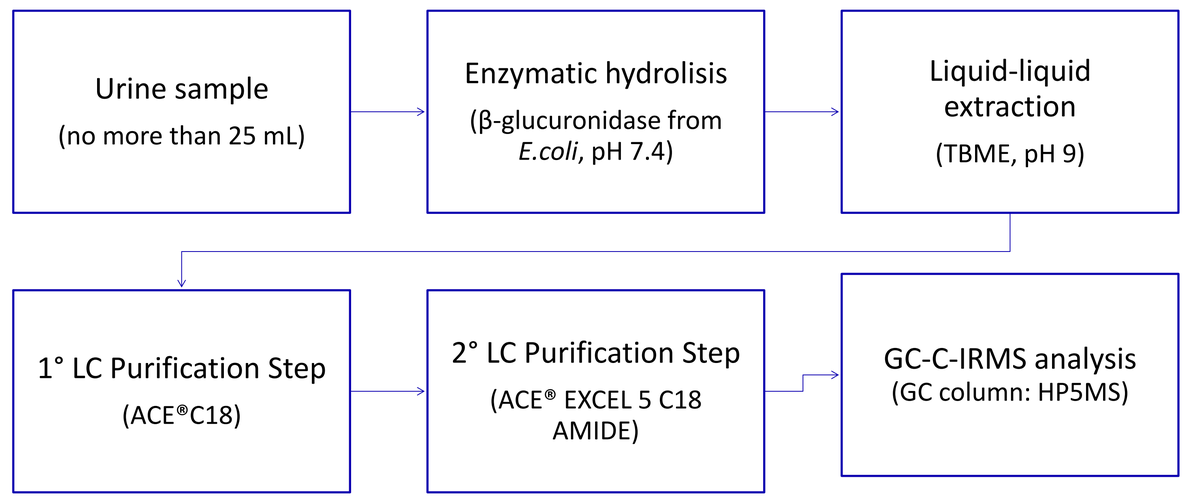
Prednisolone and its prodrug, prednisone, are two glucocorticoids widely used in clinical practice to treat inflammatory and autoimmune diseases. Because of their positive pharmacologic properties on the physical endurance and tolerance for pain, they are banned "in competition" by the World Anti-Doping Agency (WADA) when administered by intravenous, intramuscular or rectal routes[1]. A reporting level of 30 ng/mL for parent compounds and/or their metabolites has been established to disclose the permitted from the forbidden administration route. Recent studies have shown that prednisolone and prednisone could be found in urine even if no drugs administration occurred as a result of an *ex-vivo* bacterial reaction on endogenous steroids (cortisol and cortisone respectively) physiologically excreted[2-3]. WADA has established that an additional confirmatory analysis is recommended on samples in which the prednisone and/or prednisolone concentrations are between 30 and 60 ng/mL to discriminate their exogenous origin from the *ex vivo* formation.

A new method based on gas chromatography coupled to carbon isotope ratio mass spectrometry (GC-C-IRMS) was developed and fully validated according to the WADA requirements. No derivatization nor steroids structure modifications before instrumental analyses were performed. The largest urine volume (25 mL) available for the GC-C-IRMS confirmation analysis was used. The method validation parameters are discussed to prove its applicability for the analysis of real samples.



Cortisol and cortisone could be respectively converted into prednisolone and prednisone by the normal or pathogen urinary microbial flora through a Δ¹-steroid-hydrogenation (Δ¹-SDH) reaction

2 Materials and Methods



HPLC instrumental conditions

Prednisolone and prednisone were collected in the same fraction after the first HPLC clean up. A second HPLC step was developed to remove matrix interferences.

1° STEP

Time	% B
0.00	38.0
32.50	38.0
32.51	55.0
33.51	65.0
38.00	65.0
38.01	100.0
42.00	100.0

Eluents:
A. water (62 %)
B. acetonitrile (38 %)
Flow: 1 mL/min

2° STEP

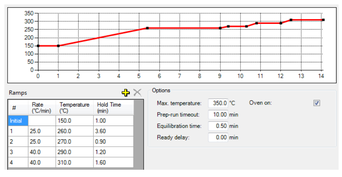
Time	% B
0.00	50.0
24.50	50.0
24.51	100.0
33.00	100.0

Eluents:
A. water (50 %)
B. methanol (50 %)
Flow: 1 mL/min

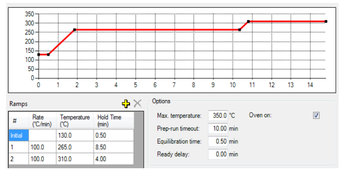
GC-C-IRMS instrumental conditions

Two different GC methods were implemented to guarantee an adequate sensitivity for both compounds.

Prednisolone GC Setting



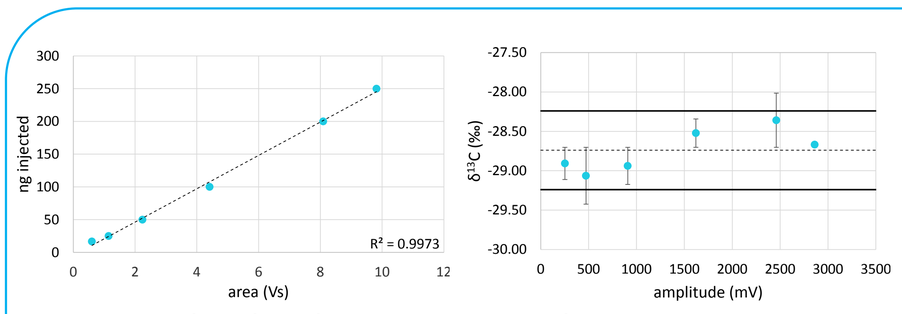
Prednisone GC Setting



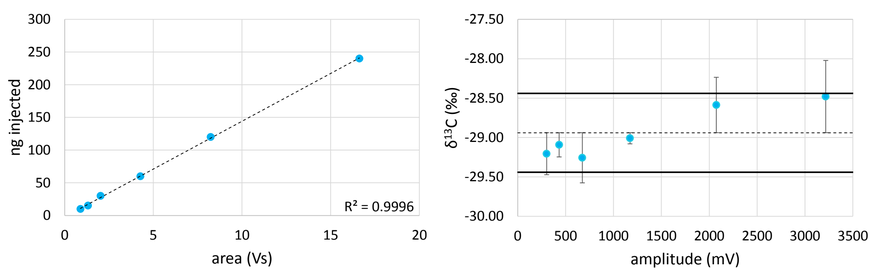
Injection temperature: 280°C
Injection mode: splitless (2 µL)

The same operating conditions were applied by injecting through a PTV (programmed temperature vaporization mode) to reduce the initial urine volume down to 10 mL.

3 Results and Discussion



Prednisolone linear response within 250 to 2860 mV corresponding to 17-250 ng injected on column. The δ¹³C (‰) average value was of -28.74 ± 0.27 ‰



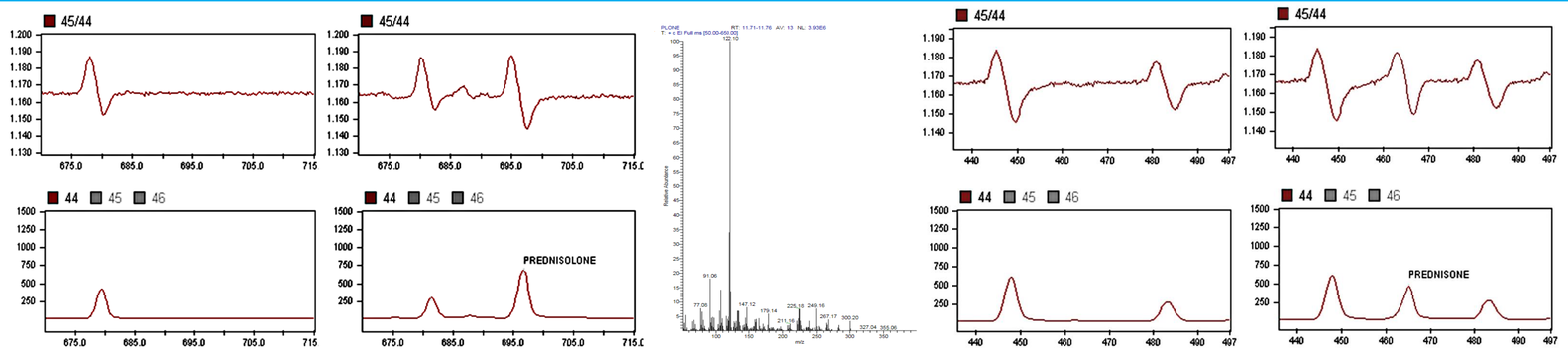
Prednisone linear response within 300 to 3200 mV corresponding to 10-240 ng injected on column. The δ¹³C (‰) average value was of -28.94 ± 0.33 ‰

Linearity

The GC-C-IRMS linearity response of prednisolone and prednisone was evaluated by injecting (2 µL splitless mode) three replicates of six scalar diluted standard solutions to define the response ranges producing consistent δ¹³C (‰) values within the instrumental linearity range (from 0.2 to 7 V).

Selectivity

Ten different blank urine and the same urine samples spiked with prednisolone and prednisone standard (30 ng/mL) were processed. In both GC methods, no interfering peaks were detected.



The GC-C-IRMS chromatograms of blank urine sample, of positive urine sample and the mass spectra of both compounds. (prednisolone on left and prednisone on right)

Repeatability

PREDNISOLONE

	δ¹³C (‰)	Ampl. (mV)
USP1	-28.69	903
USP2	-28.85	996
USP3	-28.87	542
USP4	-28.57	1353
USP5	-29.11	1516
USP6	-28.77	1402
USP7	-28.76	1526
USP8	-28.67	889
USP9	-29.14	1452
USP10	-28.81	1878
MEAN	-28.82	1246
SD	0.18	

PREDNISONE

	δ¹³C (‰)	Ampl. (mV)
USP1	-29.07	449
USP2	-28.64	466
USP3	-28.77	423
USP4	-28.71	378
USP5	-29.08	602
USP6	-28.98	383
USP7	-29.19	629
USP8	-28.97	425
USP9	-28.93	344
USP10	-28.89	483
MEAN	-28.92	458
SD	0.17	

Ten replicates of the same urine spiked (USP) at 30 ng/mL were analysed in different days. For both compounds the overall SD was < 0.20 ‰. Each δ¹³C (‰) value deviated less than 0.50 ‰ from the reference delta values extrapolated from the linearity study.

LOQ

PREDNISOLONE

	δ¹³C (‰)	Mean (n=3)	SD (n=3)	Recovery (%)
USP 30 ng/mL	-28.44	-28.54	0.24	46
	-28.37			
	-28.81			
USP 20 ng/mL	-28.89	-28.64	0.23	50
	-28.56			
	-28.46			
USP 10 ng/mL	-29.45	-29.28	0.41	55
	-28.81			
	-29.58			

PREDNISONE

	δ¹³C (‰)	Mean (n=3)	SD (n=3)	Recovery (%)
USP 30 ng/mL	-28.83	-28.89	0.14	34
	-29.05			
	-28.78			
USP 20 ng/mL	-28.68	-28.69	0.18	28
	-28.88			
	-28.52			
USP 10 ng/mL	-30.56	-30.19	0.52	25
	-29.60			
	-30.42			

Three sets of spiked urine at decreasing concentrations of prednisolone and prednisone were analysed. At 10 ng/mL the δ¹³C (‰) average value deviate from the reference value or the SD between the three measurements was not acceptable (> 0.5 ‰).

4 Conclusions

The set-up conditions allowed to obtain reproducible and reliable delta values within the linearity range down to the concentration of 20 ng/mL. The method can be applied, as required by WADA, to identify the exogenous or the *ex vivo* origin of prednisone and prednisolone.

5 References

1. WADA. International standard - The Prohibited List, https://www.wada-ama.org/en/prohibited-list
2. F. Arioli, A. Casati, M. Fidani, M. Silvestri, G. Pompa. Prednisolone and prednisone neo-formation in bovine urine after sampling. *Animal*, **2012**, *6:6*, 1023–1029.
3. M. Fidani, M. C. Gamberini, G. Pompa, F. Mungiguerra, A. Casati, F. Arioli. Presence of endogenous prednisolone in human urine. *Steroids*, **2013**, *78*, 121–126

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