Supplementary Material

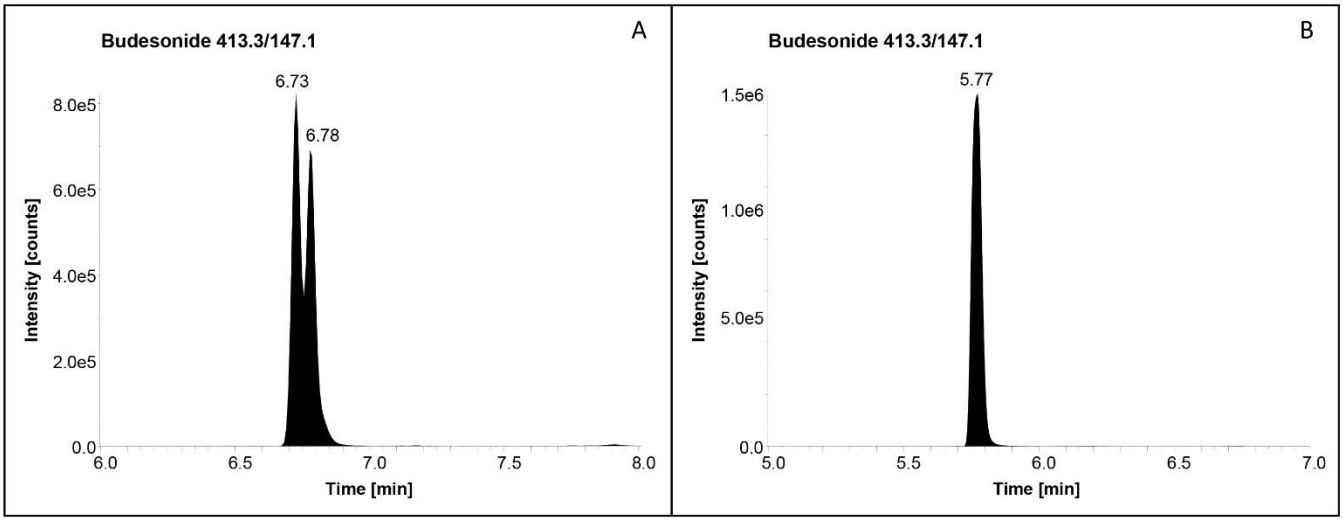

Figure S1. Extracted ion chromatograms obtained with (**A**) initial chromatographic conditions and (**B**) optimized chromatographic conditions for an extracted negative serum containing budesonide at a concentration of 10 ng/mL.

Table S1. Complete list of endogenous steroid hormones included in the mixture used for testing potential interference on analytes' selected MS transition.

Compound	Chemical Formula	Molecular Weight [Da]
11α-Hydroxyprogesterone	$C_{21}H_{30}O_3$	330.21895
11β-Hydroxyandrostenedione	$C_{19}H_{26}O_3$	302.18765
11β-Hydroxyandrosterone	$C_{19}H_{30}O_3$	306.21895
11β-Hydroxyepiandrosterone	$C_{19}H_{30}O_3$	306.21895
11β-Hydroxyetiocholanolone	$C_{19}H_{30}O_3$	306.21895
11β-Hydroxyprogesterone	$C_{21}H_{30}O_3$	330.21895
11β-Hydroxytestosterone	$C_{19}H_{28}O_3$	304.20330
11-Dehydrocorticosterone	$C_{21}H_{28}O_4$	344.19821
11-Deoxycorticosterone	$C_{21}H_{30}O_3$	330.21895
11-Deoxycortisol	$C_{21}H_{30}O_4$	346.21386
11-Ketoandrostenedione	$C_{19}H_{24}O_3$	300.17200
11-Ketoandrosterone	$C_{19}H_{28}O_3$	304.20330
11-Ketoetiocholanolone	$C_{19}H_{28}O_3$	304.20330
11-Ketotestosterone	$C_{19}H_{26}O_3$	302.18765
15β-Hydroxytestosterone	$C_{19}H_{28}O_3$	304.20330
16α-Hydroxyandrostenedione	$C_{19}H_{26}O_3$	302.18765
16α-Hydroxyandrosterone	$C_{19}H_{30}O_3$	306.21895
16α-Hydroxydehydroepiandrosterone	$C_{19}H_{28}O_3$	304.20330
16α-Hydroxyetiocholanolone	$C_{19}H_{30}O_3$	306.21895
16α-Hydroxyprogesterone	$C_{21}H_{30}O_3$	330.21895
16α-Hydroxytestosterone	$C_{19}H_{28}O_3$	304.20330
16β-Hydroxydehydroepiandrosterone	$C_{19}H_{28}O_3$	304.20330
16β-Hydroxytestosterone	$C_{19}H_{28}O_3$	304.20330

17α-Hydroxypregnenolone	$C_{21}H_{32}O_3$	332.23460
17α-Hydroxyprogesterone	$C_{21}H_{30}O_3$	330.21895
19-Hydroxyandrostenedione	$C_{19}H_{26}O_3$	302.18765
19-Hydroxytestosterone	$C_{19}H_{28}O_3$	304.20330
20α-Cortolone	$C_{21}H_{34}O_5$	366.24008
20α-Dihydrocortisone	$C_{21}H_{30}O_5$	362.20878
20α-Dihydroprogesterone	$C_{21}H_{32}O_2$	316.23968
20β-Cortolone	$C_{21}H_{34}O_5$	366.24008
21-Deoxycortisol	$C_{21}H_{30}O_4$	346.21386
21-Hydroxypregnenolone	$C_{21}H_{32}O_3$	332.23460
2β-Hydroxytestosterone	$C_{19}H_{28}O_3$	304.20330
5α-Androstane-3α,17α-diol	$C_{19}H_{32}O_2$	292.23968
5α-Androstane-3α,17β-diol	$C_{19}H_{32}O_2$	292.23968
5α-Androstane-3β,17α-diol	$C_{19}H_{32}O_2$	292.23968
5α-Androstane-3β,17β-diol	$C_{19}H_{32}O_2$	292.23968
5β-Androstane-3α,17α-diol	$C_{19}H_{32}O_2$	292.23968
5β-Androstane-3α,17β-diol	$C_{19}H_{32}O_2$	292.23968
5β-Androstane-3β,17α-diol	$C_{19}H_{32}O_2$	292.23968
5β-Androstane-3β,17β-diol	$C_{19}H_{32}O_2$	292.23968
6α-Hydroxytestosterone	$C_{19}H_{28}O_3$	304.20330
6β-Hydroxytestosterone	$C_{19}H_{28}O_3$	304.20330
7α-Hydroxydehydroepiandrosterone	$C_{19}H_{28}O_3$	304.20330
7α-Hydroxypregnenolone	$C_{21}H_{32}O_3$	332.23460
7α-Hydroxytestosterone	$C_{19}H_{28}O_3$	304.20330
7β-Hydroxydehydroepiandrosterone	$C_{19}H_{28}O_3$	304.20330
Aldosterone	$C_{21}H_{28}O_5$	360.19313
Allopregnanolone	$C_{21}H_{34}O_2$	318.25588
Allotetrahydrocortisol	$C_{21}H_{34}O_5$	366.24008
Androstenedione	$C_{19}H_{26}O_2$	286.19273
Androsterone	$C_{19}H_{30}O_2$	290.22403
Corticosterone	$C_{21}H_{30}O_4$	346.21386
Dehydroandrosterone	$C_{19}H_{28}O_2$	288.20838
Dehydroepiandrosterone	$C_{19}H_{28}O_2$	288.20838
Dihydrotestosterone	$C_{19}H_{30}O_2$	290.22403
Epitestosterone	$C_{19}H_{28}O_2$	288.20838
Etiocholanolone	$C_{19}H_{30}O_2$	290.22403
Pregnanolone	$C_{21}H_{34}O_2$	318.25533
Pregnenolone	$C_{21}H_{32}O_2$	316.23968
Progesterone	$C_{21}H_{30}O_2$	314.22403
Testosterone	$C_{19}H_{28}O_2$	288.20838
Tetrahydrocortisone	$C_{21}H_{32}O_5$	364.22443

Table S2. Calibration line equations obtained during the three days of quantitative validation protocol for all target analytes.

Compound	Calibration Line Equation		
	Day 1	Day 2	Day 3
Beclomethasone	y=0.16566x+0.02315 (R^2=0.99276)	y=0.16245x+0.02038 (R^2=0.99416)	y=0.15872x+0.02564 (R^2=0.99388)
Betamethasone	y=0.09586x+0.00067 (R^2=0.99175)	y=0.09209x+0.00065 (R^2=0.99352)	y=0.09389x+0.00061 (R^2=0.99435)
Budesonide	y=0.12123x+0.01405 (R^2=0.99332)	y=0.12458x+0.01722 (R^2=0.99518)	y=0.11982x+0.01900 (R^2=0.99614)
Cortisol	y=0.06371x+0.28312 (R^2=0.99456)	y=0.06459x+0.18898 (R^2=0.99632)	y=0.06484x+0.24131 (R^2=0.99511)
Cortisone	y=0.26142x-0.033084 (R^2=0.99231)	y=0.26366x-0.03790 (R^2=0.99489)	y=0.25929x-0.02819 (R^2=0.99656)
Dexamethasone	y=0.09079x+0.01683 (R^2=0.99721)	y=0.09241x+0.00835 (R^2=0.99374)	y=0.08818x+0.01700 (R^2=0.99456)
Flumethasone	y=0.04325x+0.00212 (R^2=0.99345)	y=0.04325x+0.00212 (R^2=0.99345)	y=0.08818x+0.01700 (R^2=0.99456)
Prednisone	y=0.00878x+0.00073 (R^2=0.99572)	y=0.00922x+0.00068 (R^2=0.99313)	y=0.00899x+0.00063 (R^2=0.99629)
Triamcinolone	y=0.02810x+0.00507 (R^2=0.99513)	y=0.02768x+0.00481 (R^2=0.99294)	y=0.02882x+0.00546 (R^2=0.99333)

Table S3. Stability of extracts stored for 3 and 7 days at 4 °C after the extraction.

	Sample	Concentration [ng/mL]				
		Day 0	Day 3	Recovery [%]	Day 7	Recovery [%]
Beclomethasone	**Val5**	28,7	28,4	99,0%	28,0	97,6%
	Val5	29,1	28,9	99,3%	28,4	97,6%
	Val5	28,9	28,3	97,9%	27,7	95,8%
	Val5	29,3	28,3	96,6%	27,4	93,5%
Betamethasone	**Val5**	25,3	24,8	98,0%	24,6	97,2%
	Val5	24,7	25,1	101,6%	24,8	100,4%
	Val5	24,3	23,9	98,4%	23,3	95,9%
	Val5	25,2	24,0	95,2%	22,9	90,9%
Budesonide	**Val5**	24,3	23,8	97,9%	23,1	95,1%
	Val5	23,9	24,0	100,4%	23,8	99,6%
	Val5	25,3	24,4	96,4%	24,0	94,9%
	Val5	22,9	22,7	99,1%	22,0	96,1%
Cortisol	**QC High**	185,2	171,3	92,5%	165,3	89,3%
	QC High	167,8	154,9	92,3%	155,8	92,8%
	QC High	170,9	165,9	97,1%	162,2	94,9%
	QC High	178,1	165,3	92,8%	165,5	92,9%
Cortisone	**QC High**	44,1	43,0	97,5%	41,6	94,3%
	QC High	42,8	42,1	98,4%	40,4	94,4%
	QC High	43,5	40,8	93,8%	41,3	94,9%
	QC High	42,3	41,4	97,9%	40,9	96,7%

Dexamethasone	**QC High**	45,3	44,8	98,9%	43,9	96,9%
	QC High	44,9	43,7	97,3%	43,0	95,8%
	QC High	43,7	44,0	100,7%	43,1	98,6%
	QC High	45,6	44,9	98,5%	44,2	96,9%
Flumethasone	**Val5**	46,2	45,0	97,4%	44,6	96,5%
	Val5	48,7	47,9	98,4%	48,1	98,8%
	Val5	51,9	50,1	96,5%	51,2	98,7%
	Val5	44,1	42,6	96,6%	42,9	97,3%
Prednisone	**Val5**	27,4	27,0	98,5%	26,7	97,4%
	Val5	28,8	28,1	97,6%	26,9	93,4%
	Val5	31,5	30,1	95,6%	28,5	90,5%
	Val5	29,9	27,4	91,6%	26,6	89,0%
Triamcinolone	**Val5**	52,7	50,3	95,4%	48,2	91,5%
	Val5	47,9	46,6	97,3%	45,0	93,9%
	Val5	50,6	51,2	101,2%	49,9	98,6%
	Val5	46,8	44,7	95,5%	44,2	94,4%

Table S4. Summary of quantitative validation protocol parameters' acceptance criteria and results obtained for all target analytes.

Parameter	Acceptance criteria	Beclomethasone	Betamethasone	Budesonide	Cortisol	Cortisone	Dexamethasone	Flumethasone	Prednisone	Trimacinolone
Selectivity	No interference (<20%LLOQ)	✓	✓	✓	✓	✓	✓	✓	✓	✓
Linearity	R^2>0.99	✓	✓	✓	✓	✓	✓	✓	✓	✓
Trueness [%]	85-115 (80-120 LLOQ)	94.2-106.3	98.8-105.4	93.6-104.9	97.8-105.5	95.6-105.6	98.6-105.9	93.2-108.4	96.6-106.3	94.8-105.0
Repeatability [%]	<15 (20 LLOQ)	6.3-9.5	5.8-8.9	7.1-10.3	5.5-8.5	4.6-8.6	6.7-9.2	6.6-9.9	5.7-8.8	7.4-10.1
Intermediate Precision [%]	<15 (20 LLOQ)	6.1-10.1	6.2-9.5	8.3-11.1	5.3-9.2	5.2-8.8	6.1-9.6	7.2-10.8	6.2-9.9	7.6-10.4
Combined Uncertainty [%]	<20	8.4-12.5	9.1-13.4	9.9-14.0	8.4-12.1	7.4-12.2	11.4-13.6	8.5-13.6	8.4-14.1	8.8-12.8
LLOQ [pg/mL]		500	500	500	1000	100	500	1000	250	1000
Robustness	No impact of minor modifications	✓	✓	✓	✓	✓	✓	✓	✓	✓
Carry-over [%]	<1 in the 1st blank afetr Cal6	0.4%	0.3%	0.3%	0.3%	0.6%	0.2%	0.4%	0.3%	0.5%
Stability of extracts [%]	85-115 of Day 0 value	93.5-97.6	90.9-100.4	94.9-99.6	89.3-94.9	94.3-96.7	95.8-98.6	96.5-98.8	89.0-97.4	91.5-98.6

Table S5. Calibration and validation samples composition (final concentration in serum).

Compound	Concentration [ng/mL]					
	Cal1/Val1	Cal2/Val2	Cal3/Val3	Cal4/Val4	Cal5/Val5	Cal6/Val6
Beclomethasone	0.5	2	7.5	15	30	75
Betamethasone	0.5	1.5	3	10	25	60
Budesonide	0.5	1.5	3	10	25	60
Cortisol	1	10	25	50	250	500
Cortisone	0.1	1	2.5	5	25	50
Dexamethasone	0.5	1.5	3	10	25	60
Flumethasone	1	5	10	25	50	100
Prednisone	0.25	1	2.5	10	30	75
Triamcinolone	1	5	10	25	50	100

Table S6. Internal Standard Mix composition (final concentration in serum).

Compound	Concentration [ng/mL]
Budesonide d8	10
Cortisol d4	15
Cortisone d8	5
Dexamethasone d3	5
Prednisone d8	10