USE OF DEUTERATED INTERNAL STANDARDS AND THEIR INFLUENCE ON QUALITATIVE AND QUANTITATIVE RESULTS OBTAINED FROM LC-MS/MS ANALYSIS IN DOPING CONTROL SAMPLES

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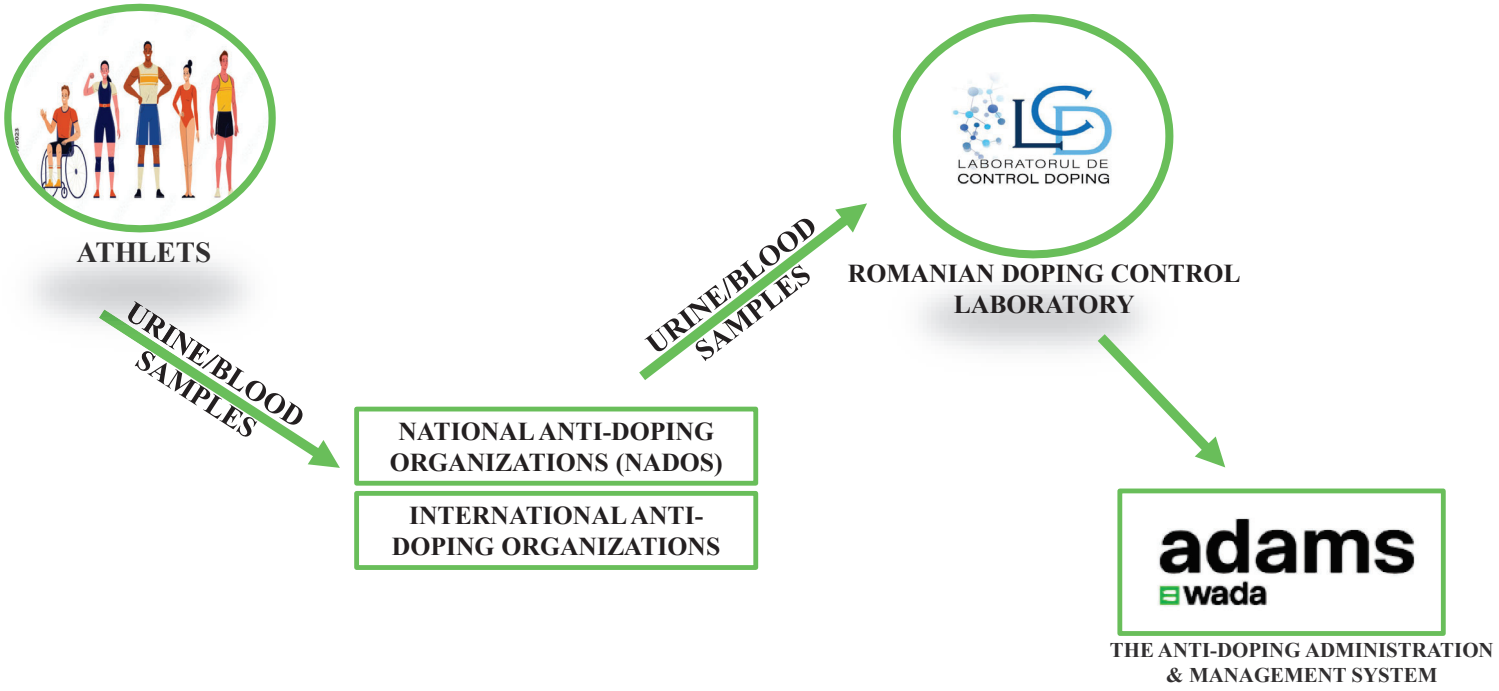
INTRODUCTION



- Initial testing (screening) procedures (ITP's):
  - qualitative
  - quantitative

- Confirmation procedures (CP's):
  - qualitative
  - quantitative

Substance Class	Threshold Substance	Threshold (T)	Maximum Relative Combined Standard Uncertainty at T Uc_Max (%)	Decision Limit (DL)
S3. Beta-2 Agonists	Salbutamol	1.00 µg/mL	10	1.20 µg/mL
	Formoterol	40 ng/mL	15	50 ng/mL
S6. Stimulants	Cathine	5.00 µg/mL	10	6.00 µg/mL
	Ephedrine	10.0 µg/mL	5	11.00 µg/mL
	Methylephedrine	10.0 µg/mL	5	11.00 µg/mL
	Pseudoephedrine	150 µg/mL	5	170.00 µg/mL
S7. Narcotics	Morphine	1.00 µg/mL	15	1.30 µg/mL

SAMPLE PROCESSING METHODS

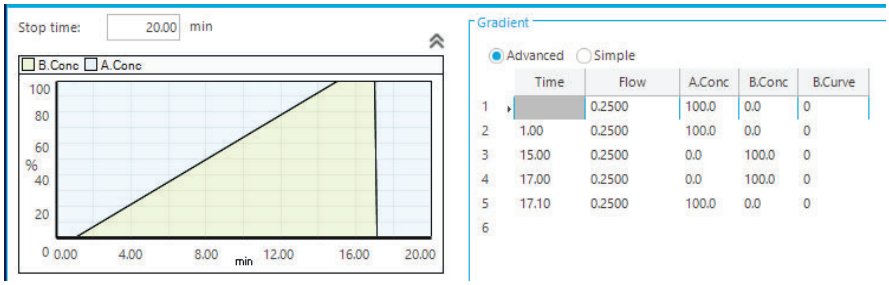
- DILUTE AND SHOOT
- LIQUID – LIQUID EXTRACTION (LLE)
- SOLID PHASE EXTRACTION (SPE)

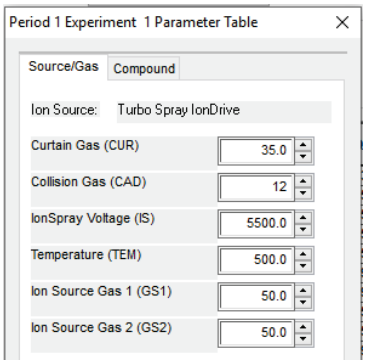
INITIAL TESTING PROCEDURE FOR FORMOTEROL – SAMPLE PREPARATION

	3 mL sample aliquot
	30 µL internal standard mix
	1mL phosphate buffer 0.8M, pH 7,0 dil 1:1 with ultrapure water
	25µL β-glucuronidase from E. Coli
	homogenisation
	Hydrolysis 1,5h at 50˚C
	1mL buffer solution K_2CO_3/$KHCO_3$ (1:1) 20%, to pH=9.6
	6mL tert-buthyl-methyl-ether
	Shake for 15 min at 135 oscilations/min
	centrifugation 10 min at 2500 rpm
	separation 2 mL organic layer
	Evaporation to dryness under nitrogen stream at 40°C±2°C
	Reconstitution with 100µL Solvent A : Methanol (30:70)
	transfer to vial

INITIAL TESTING PROCEDURE FOR FORMOTEROL – INSTRUMENTAL METHOD

- Equipment LC-MS/MS Exion LC-AD / ABSciex 6500+
- Column Zorbax SB-C18, 50 x 2.1mm, 5µm particle size
- Mobile phase: solvent A – water with 0.1% formic acid and 5mM ammonium formate; solvent B – 9:1 (v:v) acetonitrile : water with 0.1% formic acid and 5mM ammonium formate
- Acquisition mode : MRM
- Ionization: ESI Positive





CONFIRMATION PROCEDURE FOR FORMOTEROL – SAMPLE PREPARATION

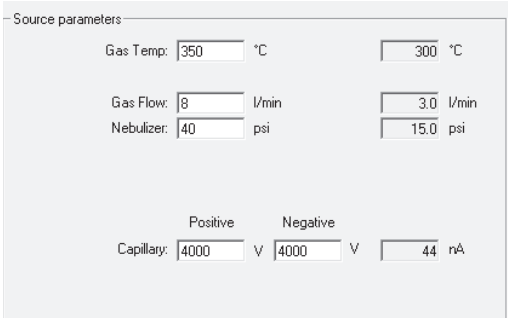
	1mL urine sample
	50µL D_6-Formoterol 2µg/mL
	1mL phosphate buffer 0.8M, pH 7,0 dil 1:1 with ultrapure water
	25µL β-glucuronidase from E. Coli
	homogenisation
	Hydrolysis 1,5h at $50^0 \pm 1^0$C
	1mL buffer $K_2CO_3/KHCO_3$ (1:1) pH=9,6
	1mL isopropanol
	5mL TBME
	Shake for 15 min at 135 oscilations/min
	centrifugation 10 min at 2500 rpm
	Separation of organic layer (discard aqueous layer)
	Evaporate to dryness at 40 ± 2^0C under nitrogen stream
	Reconstitution with 100µL mix solvent B : solvent A 1:4 (v/v)
	Transfer to vials

CONFIRMATION PROCEDURE FOR FORMOTEROL – INSTRUMENTAL METHOD

- Equipment LC-MS/MS Agilent 12160 /Agilent 6410
- Column Zorbax SB-C18, 50 x 2.1mm, 5µm particle size
- Mobile phase: solvent A – water with 0.1% formic acid and 5mM ammonium formate; solvent B – 9:1 (v:v) acetonitrile : water with 0.1% formic acid and 5mM ammonium formate
- Acquisition mode : MRM

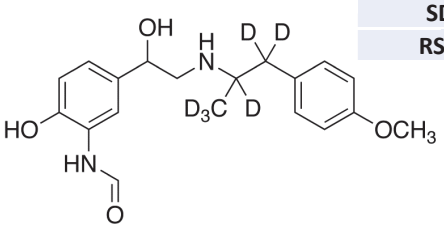
Time [min]	A [%]	B [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	90.0	10.0	0.300	400.00
2.00	60.0	40.0	0.300	400.00
5.00	35.0	65.0	0.300	400.00
5.10	90.0	10.0	0.300	400.00
9.00	90.0	10.0	0.300	400.00

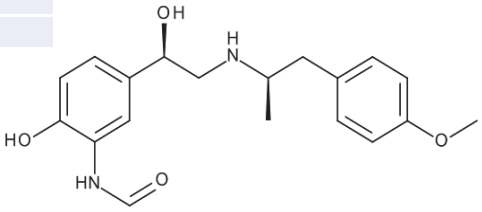
Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion
SI-D6-FORMOTEROI	☑	351	Unit	155
SI-D6-FORMOTEROI	☑	351	Unit	123
FORMOTEROL	☐	345	Unit	327
FORMOTEROL	☐	345	Unit	149
FORMOTEROL	☐	345	Unit	121
FORMOTEROL	☐	345	Unit	91



FORMOTEROL – ITP vs CP RESULTS

REPLICATE	INITIAL TESTING PROCEDURE (IS-METHYLTESTOSTERONE)	CONFIRMATION PROCEDURE (IS-D_6-FORMOTEROL)
1	43.68	39.84
2	57.65	39.79
3	53.56	39.83
4	51.2	40.02
5	45.72	40.11
6	48.46	39.65
7	52.54	40.90
8	46.34	37.51
9	43.29	39.23
10	49.2	39.81
Average	**49.16**	**39.67**
SD	**4.62**	**0.87**
RSD	**9.39**	**2.19**



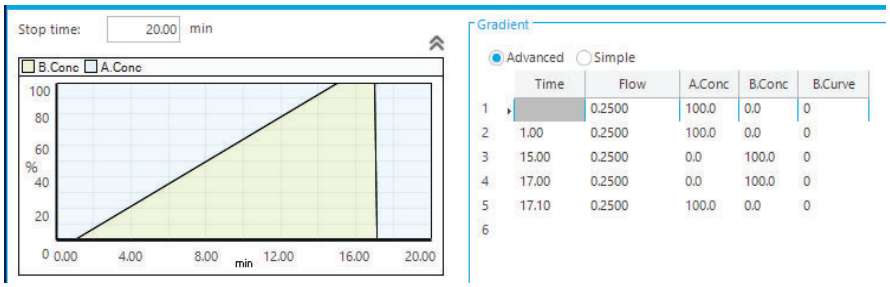


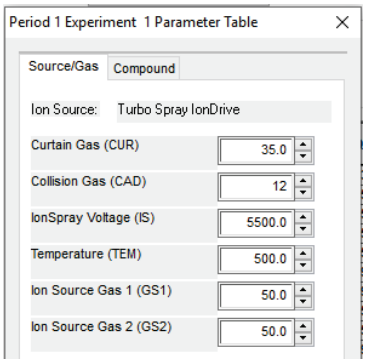
INITIAL TESTING PROCEDURE FOR MORPHINE – SAMPLE PREPARATION

	3 mL sample aliquot
	30 µL internal standard mix
	1mL phosphate buffer 0.8M, pH 7,0 dil 1:1 with ultrapure water
	25µL β-glucuronidase from E. Coli
	homogenisation
	Hydrolysis 1,5h at 50˚C
	1mL buffer solution K_2CO_3/$KHCO_3$ (1:1) to pH=9.6
	6mL tert-buthyl-methyl-ether
	Shake for 15 min at 135 oscilations/min
	centrifugation 10 min at 2500 rpm
	separation 2 mL organic layer
	Evaporation to dryness under nitrogen stream at 40°C±2°C
	Reconstitution with 100µL Solvent A : Methanol (30:70)
	transfer to vial

INITIAL TESTING PROCEDURE FOR MORPHINE – INSTRUMENTAL METHOD

- Equipment LC-MS/MS Exion LC-AD / ABSciex 6500+
- Column Zorbax SB-C18, 50 x 2.1mm, 5µm particle size
- Mobile phase: solvent A – water with 0.1% formic acid and 5mM ammonium formate; solvent B – 9:1 (v:v) acetonitrile : water with 0.1% formic acid and 5mM ammonium formate
- Acquisition mode : MRM (286>152; 286>201; 286>165)
- Ionization mode: ESI Positive



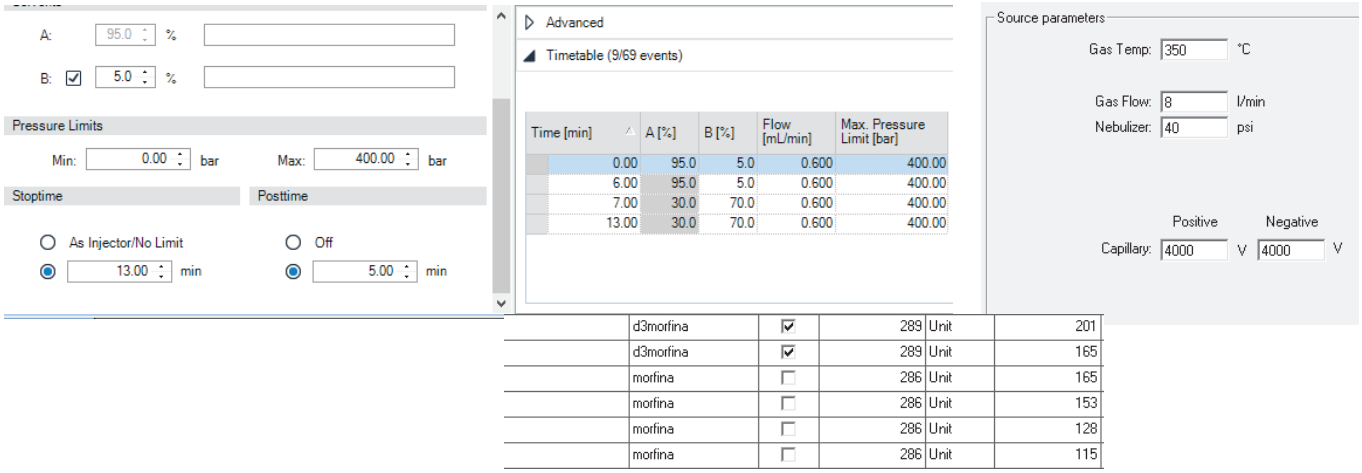


CONFIRMATION PROCEDURE FOR MORPHINE – SAMPLE PREPARATION

	2mL urine sample
	20µL SI-D_3-Morfina 50µg/mL
	0,5mL Na acetate/acetic acid 1M, pH 5,2 buffer
	50µL β-glucuronidase from Helix Pomatia
	homogenisation
	Check pH 5-5.5
	Hydrolisys 3h at 55^0C±1^0C
	500mg solid mix K_2CO_3/$KHCO_3$ (1:2)
	Check pH 9
	6mL tert-butyl-methyl-ether
	Shake for 15 min at 135 oscilations/min
	Centrifugation for 10 min at 2500rpm
	Separate organic layer (discard aqueous layer)
	Evaporation to dryness under nitrogen stream at 40°C±2°C
	Reconstitution with 100µL methanol
	Transfer to vial

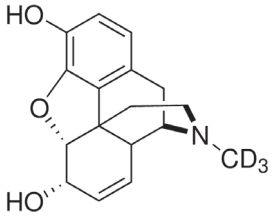
CONFIRMATION PROCEDURE FOR MORPHINE – INSTRUMENTAL METHOD

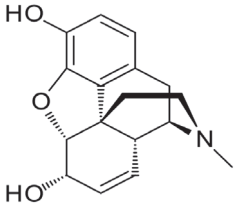
- Equipment LC-MS/MS Agilent 12160 /Agilent 6410
- Column Zorbax Eclipse XDB-C8 150x4.6mm, 5µm particle size
- Mobile phase: solvent A – water with 0.1% formic acid and 5mM ammonium formate; solvent B – 9:1 (v:v) acetonitrile : water with 0.1% formic acid and 5mM ammonium formate
- Acquisition mode : MRM Ionization mode: ESI Positive



MORPHINE – ITP vs CP RESULTS

REPLICATE	INITIAL TESTING PROCEDURE (IS-METHYLTESTOSTERONE)	CONFIRMATION PROCEDURE (IS-D_3-MORPHINE)
1	1	1.05
2	0.95	1.03
3	1.09	1.04
4	1.05	1.02
5	0.97	0.98
6	0.96	1.02
7	0.95	1.00
8	1.01	1.02
9	0.86	1.04
10	0.94	1.04
Average	**0.98**	**1.03**
SD	**0.06**	**0.02**
RSD	**6.52**	**1.93**



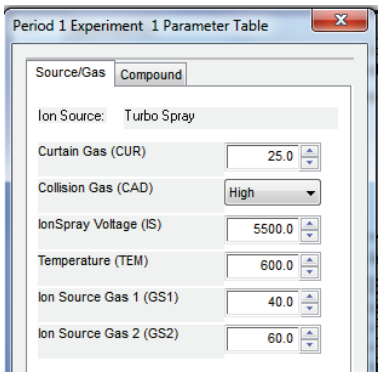


INITIAL TESTING PROCEDURE FOR SALBUTAMOL

1mL urine sample
Centrifuge for 20min at 6000 rpm
Take 40µL urine
Add 40µL solvent A (5mM ammonium formate, 0,1% formic acid in ultrapure water)
Add 20µL internal standards solution (Mefruside, and D3-Meldonium)
shake

- Equipment LC-MS/MS Agilent 1290 Infinity / ABSciex 5500QTrap
- Column Zorbax SB-C18, 50 x 2.1mm, 5µm particle size
- Mobile phase: solvent A – water with 0.1% formic acid and 5mM ammonium formate; solvent B – 9:1 (v:v) acetonitrile : water with 0.1% formic acid and 5mM ammonium formate
- Acquisition mode : MRM

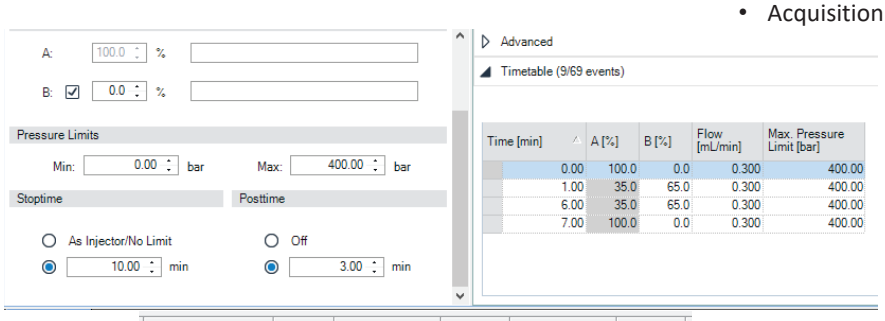
	Total Time (min)	Flow Rate (µl/min)	A (%)	B (%)
0	0.00	250	100.0	0.0
1	1.00	250	100.0	0.0
2	9.00	250	10.0	90.0
3	12.00	250	10.0	90.0
4	12.10	250	100.0	0.0
5	14.00	250	100.0	0.0

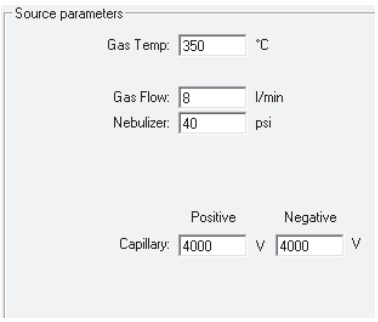


CONFIRMATION PROCEDURE FOR SALBUTAMOL

1mL urine sample
50µL SI-D_3-Salbutamol 20µg/ml
Homogenisation
Transfer to vials

- Equipment LC-MS/MS Agilent 1290 Infinity / ABSciex 5500QTrap
- Column Zorbax SB-C18, 50 x 2.1mm, 5µm particle size
- Mobile phase: solvent A – water with 0.1% formic acid and 5mM ammonium formate; solvent B – 9:1 (v:v) acetonitrile : water with 0.1% formic acid and 5mM ammonium formate
- Acquisition mode : MRM

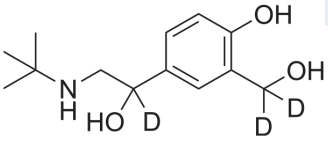


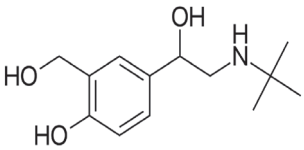


Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res
D3-SALBUTAMOL	☑	243	Unit	225	Unit
D3-SALBUTAMOL	☑	243	Unit	151	Unit
SALBUTAMOL	☐	240	Unit	222	Unit
SALBUTAMOL	☐	240	Unit	166	Unit
SALBUTAMOL	☐	240	Unit	148	Unit

SALBUTAMOL – ITP vs CP RESULTS

REPLICATE	INITIAL TESTING PROCEDURE (IS-MEFRUSIDE)	CONFIRMATION PROCEDURE (IS-D_3-SALBUTAMOL)
1	0.99	0.9599
2	0.33	1.0368
3	0.62	1.0248
4	1.12	1.0102
5	0.86	1.0317
6	0.55	1.0094
7	0.71	1.0013
8	1.43	1.0463
9	0.61	0.9905
10	0.84	1.0026
Average	**0.81**	**1.01**
SD	**0.32**	**0.03**
RSD%	**39.24**	**2.49**

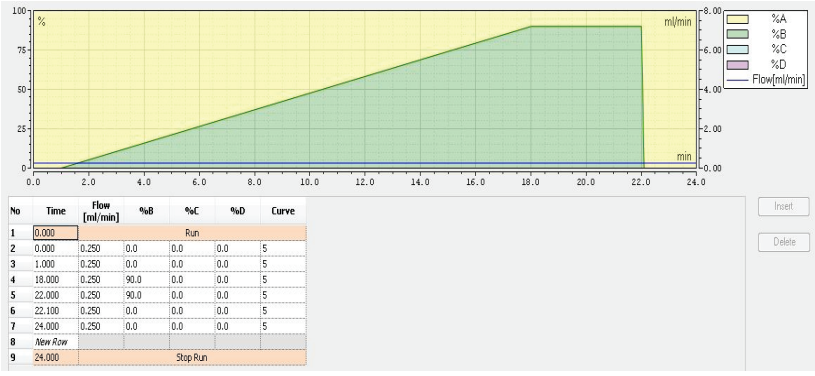




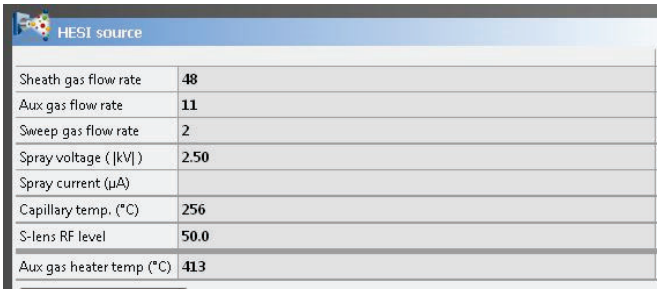
INITIAL TESTING PROCEDURE FOR EPHEDRINES

1mL urine sample
Add 10µL SI-DFA 100µg/mL
Centrifuge at 6000rpm, 5min
Take 0,25mL urine
Add 0,5mL Solvent A
Homogenise

- Equipment LC-MS/MS Dionex UliMate3000RS/ Thermo Q Exactive Plus
- Column Poroshell EC-C18, 50 x 2.1mm, 3µm particle size
- Mobile phase: solvent A – water with 0.1% formic acid and 5mM ammonium formate; solvent B – 9:1 (v:v) acetonitrile : water with 0.1% formic acid and 5mM ammonium formate
- Acquisition mode : PRM (MS/MS)



No	Time	Flow [ml/min]	%B	%C	%D	Curve
1	0.000	Run				
2	0.000	0.250	0.0	0.0	0.0	5
3	1.000	0.250	0.0	0.0	0.0	5
4	18.000	0.250	90.0	0.0	0.0	5
5	22.000	0.250	90.0	0.0	0.0	5
6	22.100	0.250	0.0	0.0	0.0	5
7	24.000	0.250	0.0	0.0	0.0	5
8	New Row					
9	24.000	Stop Run				



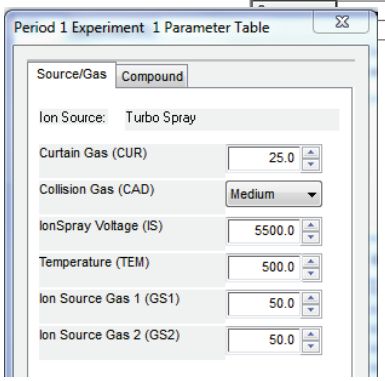
CONFIRMATION PROCEDURE FOR EPHEDRINES

0,5 mL urine
10µL SI:D_3-Efedrina 200µg/mL
0,5mL solvent A (5mM NH_4HCOO, 0,1% HCOOH)
20mL formic acid
Shake 10min. (100 oscil/min)
Transfer to vials

- Equipment LC-MS/MS Agilent 1290 Infinity / ABSciex 5500QTrap
- Betasil fenil, 250 x 4.6mm, 3µm particle size
- Mobile phase: solvent A – water with 0.1% formic acid and 5mM ammonium formate; solvent B – 9:1 (v:v) acetonitrile : water with 0.1% formic acid and 5mM ammonium formate
- Acquisition mode : MRM

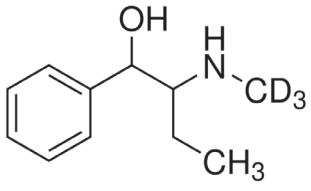
	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	ID	DP (volts)	CE (volts)	CXP (volts)
1	169.040	151.100	50.0	SI-D3-EFEDRINA	46.000	15.000	12.000
2	169.040	115.000	50.0	SI-D3-EFEDRINA	46.000	33.000	12.000
3	166.131	148.100	50.0	EFEDRINA	11.000	17.000	14.000
4	166.131	117.100	50.0	EFEDRINA	11.000	25.000	8.000
5	166.131	133.100	50.0	EFEDRINA	11.000	27.000	12.000
6	166.131	115.100	50.0	EFEDRINA	11.000	37.000	12.000
7	166.131	91.000	50.0	EFEDRINA	11.000	39.000	10.000
8	166.083	148.100	50.0	PSEUDOEFEDRINA	21.000	15.000	12.000
9	166.083	133.100	50.0	PSEUDOEFEDRINA	21.000	27.000	14.000
10	166.083	117.100	50.0	PSEUDOEFEDRINA	21.000	25.000	14.000
11	166.083	115.000	50.0	PSEUDOEFEDRINA	21.000	33.000	10.000
12	166.083	91.000	50.0	PSEUDOEFEDRINA	21.000	43.000	10.000
13	133.998	117.100	50.0	CATINA	101.000	21.000	14.000
14	133.998	115.000	50.0	CATINA	101.000	29.000	10.000
15	133.998	91.100	50.0	CATINA	101.000	35.000	12.000
16	133.998	65.100	50.0	CATINA	101.000	51.000	8.000
17	180.112	162.100	50.0	METILEFEDRINA	71.000	17.000	16.000
18	180.112	147.100	50.0	METILEFEDRINA	71.000	29.000	16.000
19	180.112	91.000	50.0	METILEFEDRINA	71.000	39.000	12.000
20	180.112	115.100	50.0	METILEFEDRINA	71.000	37.000	12.000

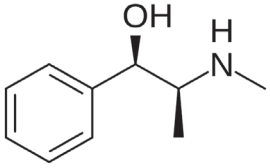
	Total Time (min)	Flow Rate (µl/min)	A (%)	B (%)
0	0.00	700	88.0	12.0
1	20.00	700	88.0	12.0



EPHEDRINE – ITP vs CP RESULTS

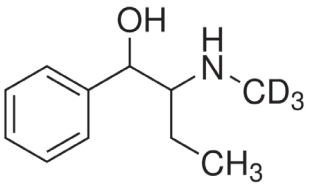
REPLICATE	INITIAL TESTING PROCEDURE (SI-DIPHENYLAMINE)	CONFIRMATION PROCEDURE (SI-D_3-EPHEDRINE)
1	12.15	9.63
2	10.38	10.52
3	11.12	9.80
4	10.86	10.27
5	10.74	10.43
6	10.33	9.86
7	10.62	10.84
8	11.3	9.02
9	9.92	10.57
10	10.74	10.07
Average	**10.82**	**10.10**
SD	**0.61**	**0.54**
RSD%	**5.67**	**5.34**

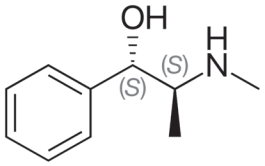




PSEUDOEPHEDRINE – ITP vs CP RESULTS

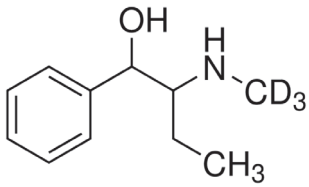
REPLICATE	INITIAL TESTING PROCEDURE (SI-DIPHENYLAMINE)	CONFIRMATION PROCEDURE (SI-D_3-EPHEDRINE)
1	153.43	162.61
2	147.94	155.47
3	136.36	158.54
4	152.11	155.92
5	150.75	154.60
6	153.4	157.75
7	144.33	160.98
8	146.74	162.82
9	152.4	147.53
10	155.11	156.07
MEDIE	**149.26**	**157.23**
SD	**5.65**	**4.51**
RSD%	**3.79**	**2.87**

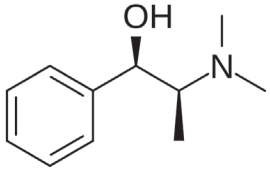




METHYLEPHEDRINE – ITP vs CP RESULTS

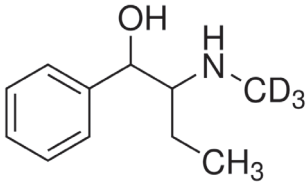
REPLICATE	INITIAL TESTING PROCEDURE (SI-DIPHENYLAMINE)	CONFIRMATION PROCEDURE (SI-D_3-EPHEDRINE)
1	9.99	8.89
2	8.37	9.08
3	9.13	9.87
4	9.49	9.33
5	9.27	9.82
6	8.00	8.93
7	8.37	9.19
8	9.90	8.88
9	8.30	10.21
10	9.46	9.09
Average	**9.03**	**9.33**
SD	**0.72**	**0.47**
RSD%	**7.93**	**5.05**

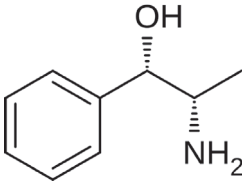




CATHINE – ITP vs CP RESULTS

REPLICATE	INITIAL TESTING PROCEDURE (IS-DIPHENYLAMINE)	CONFIRMATION PROCEDURE (IS-D_3-EPHEDRINE)
1	6.70	5.86
2	5.77	5.67
3	5.52	5.68
4	4.50	5.87
5	4.68	5.69
6	5.52	5.61
7	4.31	5.48
8	5.53	5.55
9	4.53	5.62
10	4.60	5.51
Average	**5.17**	**5.66**
SD	**0.76**	**0.13**
RSD%	**14.77**	**2.34**





CONCLUSIONS

- Deuterated internal standards are an important tool for quantitative analysis
- Because they have identical chemical structure and chemical properties as the target analytes their use leads to lower biases and lower spreads of the results, which in the end leads to lower uncertainties for quantitative determinations