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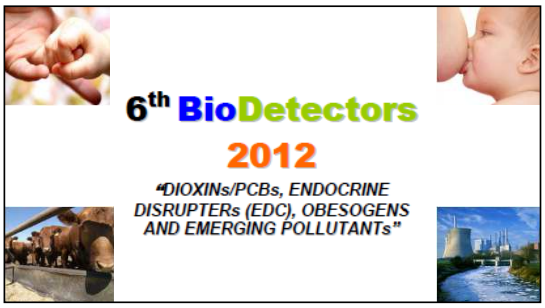
Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta

S.C. Istopatologia e Test Rapidi

GR CALUX in illegal treatment detection: from targeted to untargeted screening strategy

PRELIMINARY



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IZSTO is under the Ministery of Health

Veterinary Public Health

Our activities are focused on

1. **Control..** foodstuffs, feedingstuffs and live animals

2. **Detection..**
   contaminats
   drug residues
   agents of zoonoses
   animal diseases

3. **Research..**

Synthetic glucocorticoids

- They are widely used as therapeutic agents in veterinary practice; metabolic diseases, shock, stress and inflammatory disorders
- In some European Countries they are also utilized illegally as growth promoters, either alone or in association with anabolic steroids

Our activity in live animals

1. Correct use as therapeutic agents; respect of withdrawal time
2. Illicit treatment of producing animals

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Synthetic glucocorticoids

Testing activity in live animals

Urine samples → Screening analysis (ELISA)

(above 2 ppb) → Confirmatory analysis (LC-MS/MS)

(-) → STOP

What's the matter?
Both screening and confirmatory analyses are limited to a short list of known molecules….

What's the matter?

ELISA Specificity

Compound	% Cross-Reactivity
Dexamethasone	100
Dexamethasone 21 Acetate	162
Dexamethasone 21 Phospate	3.6
Flumethasone	50
Betamethasone	43
Betamethasone 21 Acetate	73
Betamethasone 21 Phosphate	2.0
Triamcionlone	1.0
Prednisolone	1.7
Cortisol	<0.5
Cortisone	<0.5

ELISA Limits of Detection

	Urine	Urine (IAC)
Dexamethasone	2.5 ng/ml	0.1 ng/ml
Flumethasone	5.0 ng/ml	0.2 ng/ml
Betamethasone	5.8 ng/ml	0.25 ng/ml

LC-MS/MS analysis: simultaneous detection of six glucocorticoids (dexamethasone, flumethasone, betamethasone, prednisolone, prednisone, methylprednisolone).

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GR-CALUX

- Detection based on the glucocorticoid activity of each molecule, not on the "signature" of each molecule
- Potentially it could detect every molecule with glucocorticoid activity at a very low level
- Unimportant effect of natural glucocorticoids

Study design
1. Blank samples (CALUX, ELISA, LC-MS/MS)
2. Pure compouds (EC50, REP)
3. Spiked samples (CALUX, ELISA, LC-MS/MS)

3 Partners: TEST VERITAS, IZSTO, BDS

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1. Blank samples I

20 urine samples (calves)
animals bred under control conditions
samples collected at farm

IZSTO: samples and LC- MS/MS natural GCs

VERITAS: ELISA **BDS:** CALUX

Screening positive 2 ppb

	LC-MS/MS analysis (ppb)					GR CALUX	ELISA
Sample	cortisol	cortisone	20β-dihydrocortisol	6β-hydroxycortisol	TOT.	ng desa eq/ml	(ppb)
08/06	3,1	5,3	1,3	nr	9,8	0,73	0,31
08/10	1,3	2,3	0,9	nr	4,5	<LOD (0,078)	1,23
08/21	15,7	15,4	9,5	14,1	54,6	3,7	2,96
08/26	nr	0,8	nr	nr	0,8	<LOD (0,078)	<0,250
08/45	0,5	1,0	0,6	nr	2,1	<LOD (0,078)	0,95
08/68	nr	0,7	nr	nr	0,7	<LOD (0,078)	0,50
08/71	nr	nr	nr	6,7	6,7	0,68	0,61
08/72	1,1	1,5	0,6	nr	3,2	0,31	0,50
08/79	nr	0,6	nr	nr	0,6	0,34	0,49
08/88	0,6	1,0	0,9	nr	2,6	<LOD (0,078)	0,84
09/06	0,6	nr	1,3	nr	1,9	<LOD (0,078)	1,98
09/31	nr	nr	1,4	7,9	9,3	<LOD (0,078)	1,70
09/32	1,4	0,7	1,4	nr	3,5	<LOQ (0,21)	1,48
09/35	1,4	1,1	0,8	nr	3,2	<LOD (0,078)	2,62
09/36	0,8	1,7	1,0	nr	3,6	0,95	1,89
09/38	nr	0,5	1,4	nr	2,0	<LOD (0,078)	1,78
09/41	1,4	1,2	1,2	nr	3,9	<LOD (0,078)	1,32
09/42	2,0	1,5	2,5	nr	6,1	1,1	1,93
09/43	1,3	1,5	1,6	nr	4,4	0,44	1,99
09/44	0,7	1,0	1,2	nr	2,9	0,33	1,37

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1. Blank samples II

3 urine samples (bulls)
animals bred under control conditions
samples collected at slaughterhouse

IZSTO: samples and LC- MS/MS natural GCs

VERITAS: ELISA **BDS:** CALUX

Screening positive 2 ppb

	LC-MS/MS (ppb)					GR CALUX	ELISA
Sample	cortisol	cortisone	20β-dihydrocortisol	6β-hydroxycortisol	TOT.	ng desa eq/ml	(ppb)
6196	30,0	33,6	29,0	48,0	140,6	25	4,64
6210	4,7	5,7	2,9	5,2	18,6	3,3	0,58
9446	25,5	17,4	24,7	14,8	82,4	10	2,28

VS

2. Pure compounds

BDS evaluated the EC50 and REP of the most used synthetic GCs

VERITAS provided the standards

Mandatory detection capability 2 ppb

Compound	EC50 (ppb)	REP relative to dex VERITAS	REP relative to dex BDS
Prednisolone	2.3	0.52	0.21
Prednisone	n.a.	n.a.	n.a.
Methylprednisolone	0.99	1.23	0.48
Betamethasone	0.57	2.14	0.84
Dexamethasone	1.2	1.00	0.39
Flumethasone	0.13	9.68	3.81
Dexamethasone (BDS)	0.48		1.00

Difference between dex BDS and dex VERITAS
Probably due to different formulation (acetate, phosfate)
..under investigation..

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3. Spiked samples

IZSTO samples

VERITAS spiking

BDS CALUX analysis

Compound	1 ppb (1/2 CCβ)	2 ppb (CCβ)	4 ppb (2 CCβ)
Prednisolone			
Methylprednisolone			
Betamethasone			
Dexamethasone			
Flumethasone			

CONCLUSIONS

PRELIMINARY

1. The physiological levels of endogenous GCs don't seem to influence CALUX measurements
2. At the slaughterhouse the increase in GCs levels could invalidate the analysis
3. GR CALUX showed, using pure compounds, a sensitivity lower than the mandatory detection capability (CCβ), comparable with a confirmatory test
4. Prohormones can't be detected by GR CALUX (prednisone)
5. The technique looks a promising screening tool for the detection of illicit treatments in animals but more focused experiments are needed

NEXT STEP.. *GR CALUX validation as screening test for GCs detection in bovine urine sample*

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Thank you

Acknowledgments

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