Recent Advancements in Analytical Methods of Drug Detection

Mario Thevis

WORLD ANTI-DOPING AGENCY
play true

The World Anti-Doping Code

THE 2013 PROHIBITED LIST

INTERNATIONAL STANDARD

WORLD ANTI-DOPING AGENCY
play true

S0. NON-APPROVED SUBSTANCES

S1. ANABOLIC AGENTS

S2. PEPTIDE HORMONES, GROWTH FACTORS AND RELATED SUBSTANCES

S3. BETA-2 AGONISTS

S4. HORMONE AND METABOLIC MODULATORS

S5. DIURETICS AND OTHER MASKING AGENTS

M1. MANIPULATION OF BLOOD AND BLOOD COMPONENTS

M2. CHEMICAL AND PHYSICAL MANIPULATION

M3. GENE DOPING

S6. STIMULANTS

S7. NARCOTICS

S8. CANNABINOIDS

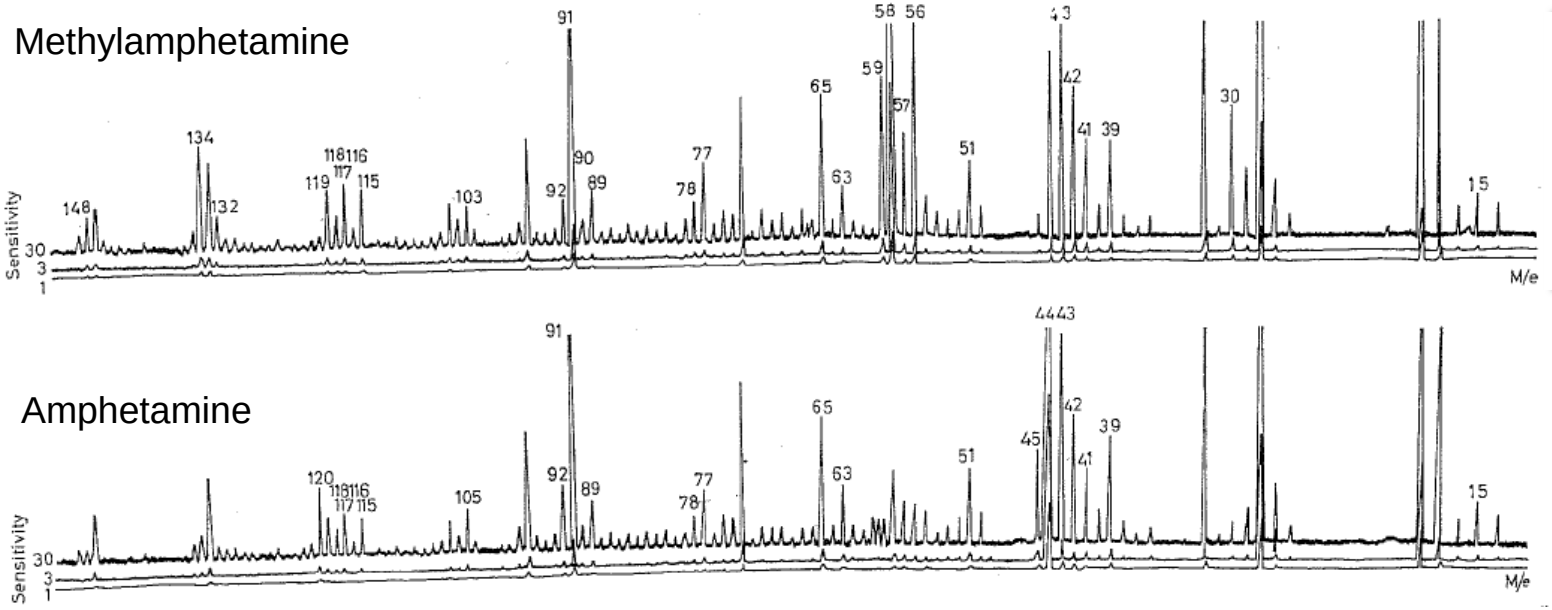
S9. GLUCOCORTICOSTEROIDS

P1. ALCOHOL

P2. BETA-BLOCKERS

Challenges at the time accepted by anti-doping authorities and sports drug testing laboratories

- use of state-of-the-art instrumentation
- steep learning curve concerning analytical methodologies



Hitachi RMU 6E

12s/scan

LOD: 4 µg/mL

Main tools have always been chromatography / mass spectrometry

- Monopoly of gas chromatography – mass spectrometry until early 2000

  *Limitation: only volatile substances can be measured / extensive derivatization required

  *Advantage: robustness, reproducibility, steroid profiling, IRMS

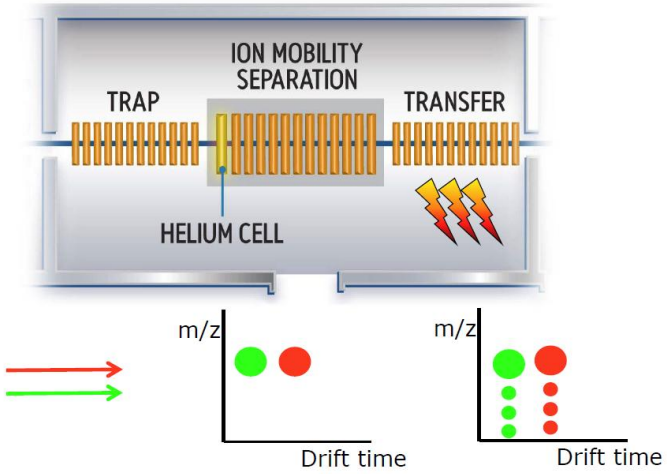
Main tools have always been chromatography / mass spectrometry

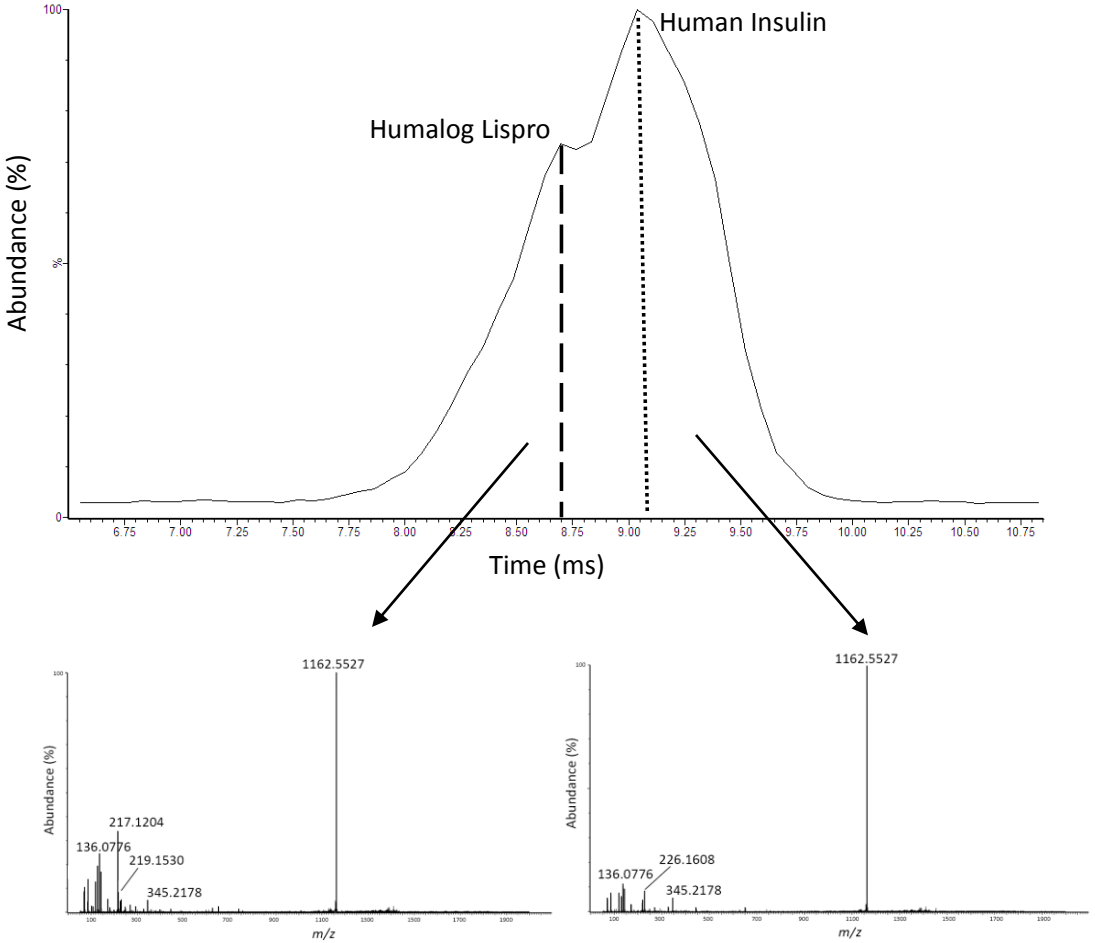
- Availability of new generation liquid chromatography – mass spectrometry instruments

  *Peptides, proteins, carbohydrates as well as nucleotides can be measured
  *commonly no / little derivatization and sample preparation required

- Substantial improvements in resolution and mass accuracy

Main tools have always been chromatography / mass spectrometry





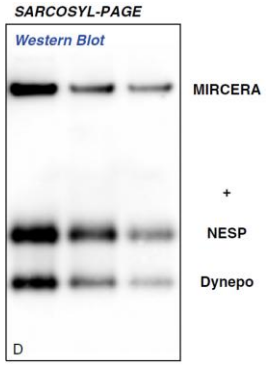
Complementary methods

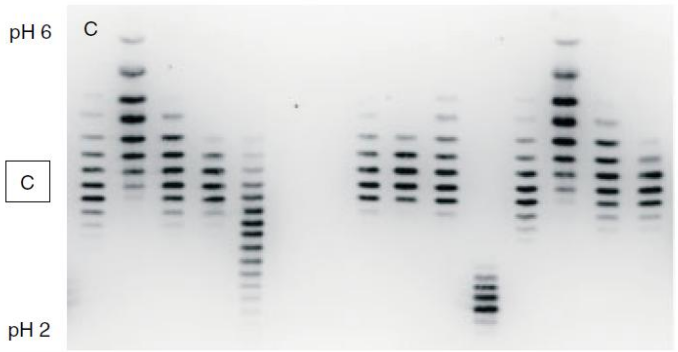
- Immunological methods

  (e.g. hCG, LH, hGH)

- 1- and 2-D electrophoretic/immunological methods

  (e.g. EPO, proteases)





Combined methods

- Immunopurification for MS-based methodologies
  (e.g. insulins, GHRH, LHRH, CRH)

- SPE for LC-MS/MS-based methodologies
  (e.g. GHRPs, TB-500, AOD-9604, LHRH)

- Bottom-up targeted proteomics approaches
  (e.g. IGF-1, hematide)

- Bottom-up targeted `RNomics´ approaches
  (e.g. siRNA)

Currently: detection assays composed by methodology

GC-MS	LC-MS(/MS)	Complementary
S0. NON-APPROVED SUBSTANCES	S0. NON-APPROVED SUBSTANCES	M1. MANIPULATION OF BLOOD AND BLOOD COMPONENTS
S1. ANABOLIC AGENTS	S1. ANABOLIC AGENTS	M3. GENE DOPING
S6. STIMULANTS	M3. GENE DOPING	S2. PEPTIDE HORMONES
S7. NARCOTICS	S2. PEPTIDE HORMONES	
P1. ALCOHOL	S3. BETA-2 AGONISTS	
	S4. HORMONE AND METABOLIC MODULATORS	
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	M2. CHEMICAL AND PHYSICAL MANIPULATION	
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	S8. CANNABINOIDS	
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	P2. BETA-BLOCKERS	

Journal of Chromatography A, 1216 (2009) 4423–4433

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Fast analysis of doping agents in urine by ultra-high-pressure liquid chromatography–quadrupole time-of-flight mass spectrometry I. Screening analysis

F. Badoud[a,b,c], E. Grata[a,b,c], L. Perrenoud[a,c], L. Avois[a,c], M. Saugy[a,c], S. Rudaz[b,c], J.-L. Veuthey[b,c,*]

[a] Swiss Anti Doping Laboratory, University Center of Legal Medicine, Geneva and Lausanne, Chemin des Croisettes 22, 1066 Epalinges, Switzerland
[b] School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 20 Bd d'Yvoy, CH-1211 Geneva 4, Switzerland
[c] Swiss Center of Applied Human Toxicology, University of Geneva, CMU, 1 Rue Michel-Servet, CH-1206 Geneva, Switzerland

JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2008; **43**: 980–992
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(www.interscience.wiley.com) DOI: 10.1002/jms.1436

JMS

A high-throughput multicomponent screening method for diuretics, masking agents, central nervous system (CNS) stimulants and opiates in human urine by UPLC–MS/MS

John-Olof Thörngren,* Fredrik Östervall and Mats Garle

Doping Control Laboratory, Karolinska University Hospital, Huddinge, Sweden

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Research article

Drug Testing and Analysis

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(wileyonlinelibrary.com) DOI 10.1002/dta.372

High-throughput screening for various classes of doping agents using a new 'dilute-and-shoot' liquid chromatography-tandem mass spectrometry multi-target approach

S. Guddat,[a]* E. Solymos,[b] A. Orlovius,[a,c] A. Thomas,[a] G. Sigmund,[a] H. Geyer,[a] M. Thevis[a] and W. Schänzer[a]

Journal of Chromatography A, 1288 (2013) 82–95

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Use of ultra-high pressure liquid chromatography coupled to high resolution mass spectrometry for fast screening in high throughput doping control

Alessandro Musenga, David A. Cowan*

Drug Control Centre, King's College London, London SE1 9NH, UK

Modern mass spectrometry-based detection assay

Major advantages:

- Combined targeted AND non-targeted analyses
- Retrospective data mining
- Comprehensive coverage of most prohibited substances
- Structure-based identification of related compounds
- Determination of elemental composition

Instrumentation and methodologies principally available in all accredited doping control laboratories!

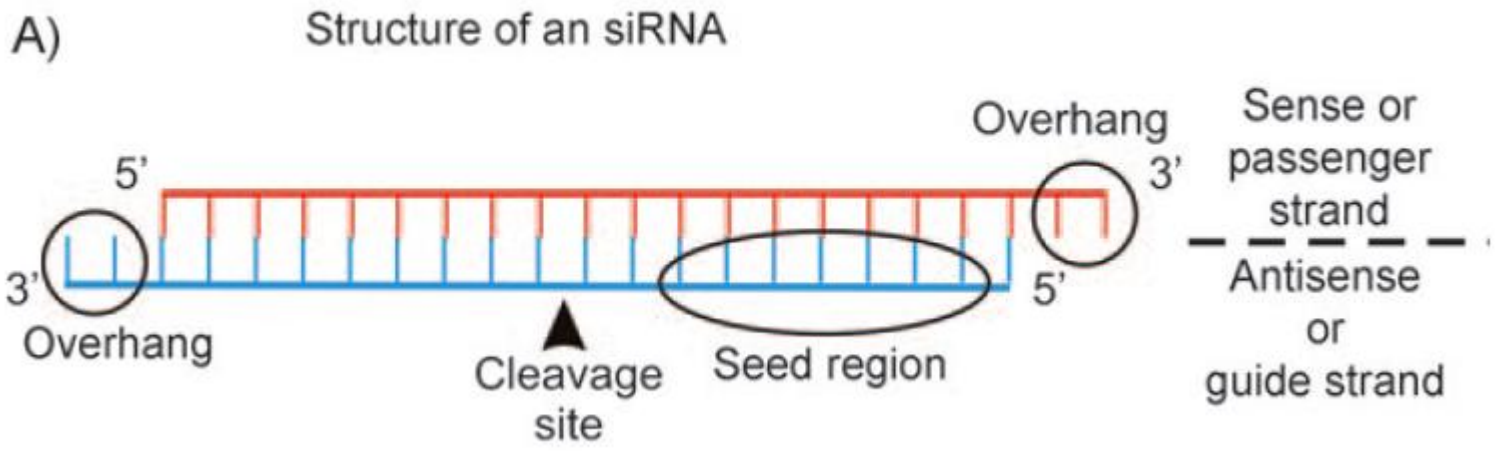
Modern mass spectrometry-based detection assay

Recent advances - Example

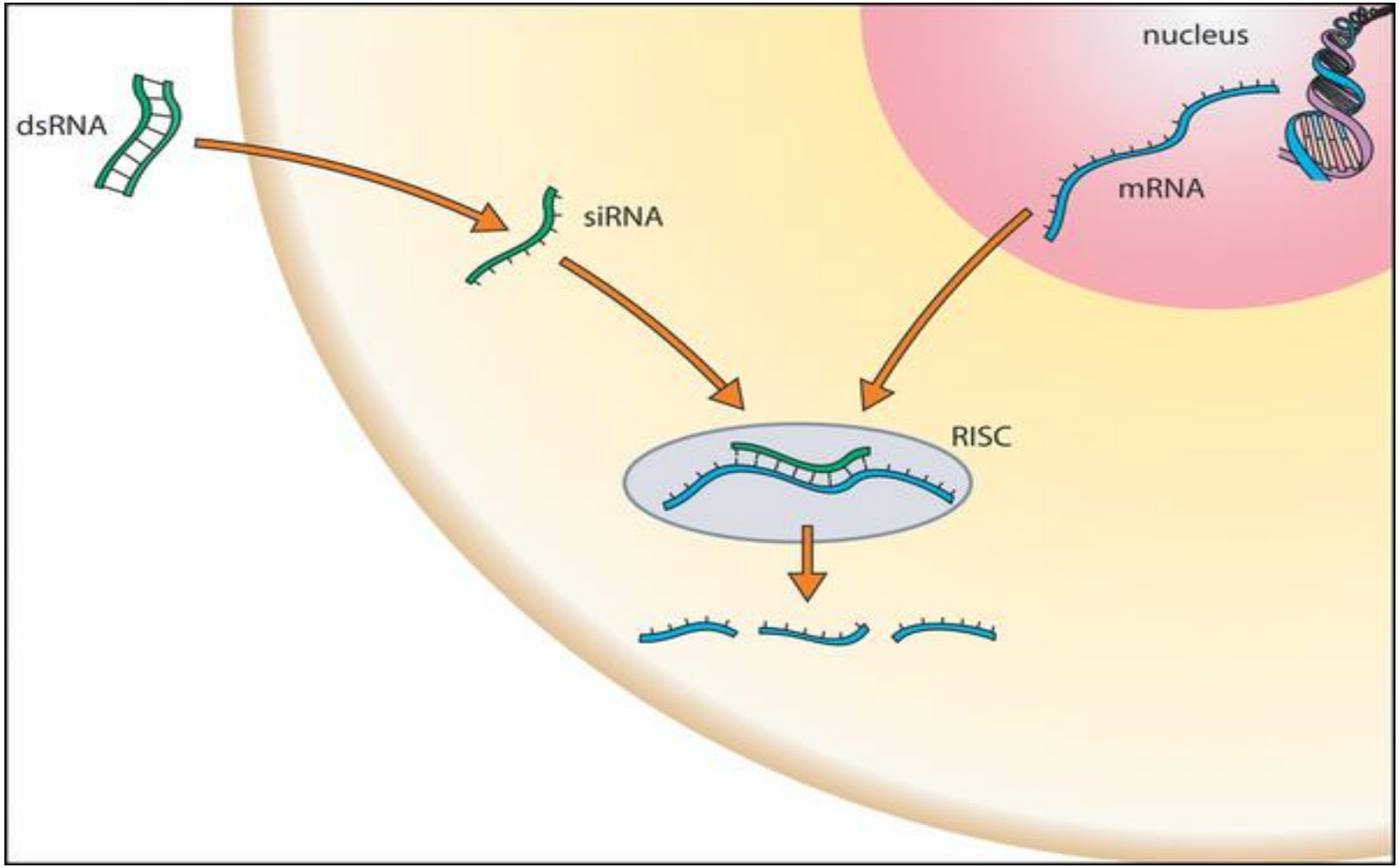
M3. GENE DOPING

The following, with the potential to enhance sport performance, are prohibited:

1. The transfer of polymers of nucleic acids or nucleic acid analogues;
2. The use of normal or genetically modified cells.



Small Interfering RNA (siRNA)



From: RNAi Therapeutics: How Likely, How Soon? Robinson R PLoS Biology Vol. 2, No. 1, e28 doi:10.1371/journal.pbio.0020028

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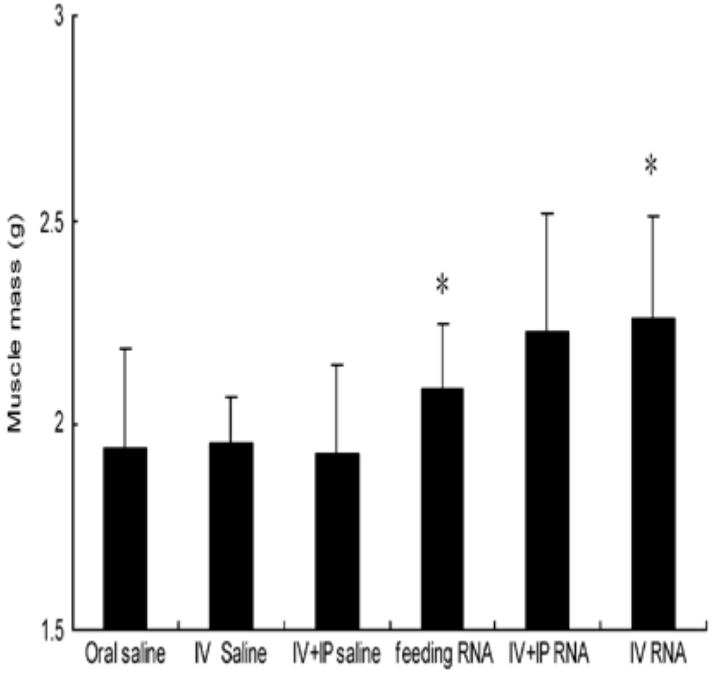

Gene Therapy (2008) 15, 155–160

www.nature.com/gt

ORIGINAL ARTICLE

Myostatin antisense RNA-mediated muscle growth in normal and cancer cachexia mice

C-M Liu[1,3], Z Yang[1,3], C-W Liu[1], R Wang[1], P Tien[1], R Dale[2] and L-Q Sun[2]
[1]*Molecular Virology Research Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China and* [2]*Oligos Etc Inc., Wilsonville, OR, USA*

Myostatin is a negative regulator of myogenesis, and inactivation of myostatin leads to muscle growth. Here we have used modified RNA oligonucleotides targeting the myostatin mRNA and examined the therapeutic potential in normal and cancer cachexia mouse models. We found that the RNA oligonucleotides could suppress the myostatin expression in vivo, *leading to the increase in muscle growth both in normal and cachectic mice. We also established that the effect of myostatin inhibition caused by the RNA oligonucleotides may be through the MyoD pathway, as evidenced by a significant upregulation of MyoD expression. Taken together, these results demonstrate the feasibility using antisense strategy for the treatment of muscle wasting conditions.*
Gene Therapy (2008) **15**, 155–160; doi:10.1038/sj.gt.3303016; published online 22 November 2007

Keywords: *myostatin; antisense; muscle wasting; cancer cachexia*

WORLD ANTI-DOPING AGENCY
WADA-supported project

Model siRNA designed from the myostatin mRNA of *Rattus norvegicus*

ATGATTCAAAAACCGCAAATGTATGTTTATATTTACCTGTTTGTGCTGATTGCTGCTGGCCCAG
TGGATCTAAATGAGGACAGTGAGACAGAGGCGAATGTGGAAAAAGAGGGGCTGTGTAATGCG
TGTGCGTGGAGACAAAACACAAGGTACTCCAGAATAGAAGCCATAAAAATTCAAATCCTCAGT
AAACTCCGCCTGGAAACAGCGCCTAACATCAGCAAAGATGCTATAAGACAACTTCTGCCCAGA
GCGCCTCCACTCCGGGAACTGATCGATCAGTACGACGTCCAGAGGGATGACAGCAGTGACG
GCTCTTTGGAAGATGACGATTATCACGCTACCACGGAAACAATCATTACCATGCCTACCGAGT
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ACAGTACAACAAAGTGGTAAAGGCCCAGCTGTGGATATATCTGAGAGCCGTCAAGACTCCTAC
AACAGTGTTTGTGCAAATCCTGAGACTCATCAAACCCATGAAAGACGGTACAAGGTATACCGG
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CCTTTTTAGAAGTCAAAGTAACAGACACACCCAAGAGGTCCCGGAGAGACTTTGGGCTTGACT
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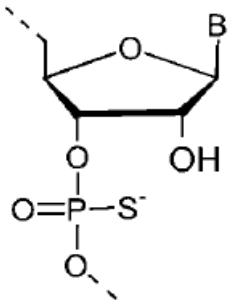
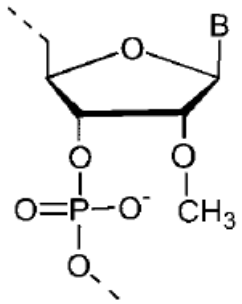
TTTGGATGGGACTGGATTATTGCACCCAAAAGATATAAGGCTAATTACTGCTCTGGAGAGTGT
GAATTTGTGTTCTTACAAAAATATCCGCATACTCATCTTGTGCACCAAGCAAACCCCAGAGGCT
CGGCAGGCCCTTGCTGCACGCCAACAAAAATGTCTCCCATTAATATGCTATATTTTAATGGCAA
AGAACAAATAATATATGGGAAAATTCCAGCCATGGTAGTAGACCGGTGTGGGTGCTCGTGA

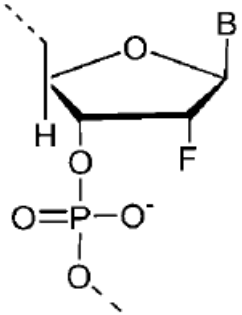
Selection of RNA nucleotide modifications



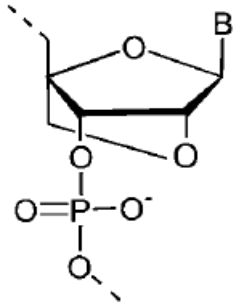
Phosphorothioate-RNA
(PS)



2'-*O*-Methyl-RNA
(OMe)



2'-Fluoro-
nucleotide



Locked nucleic
acid (LNA)

In vivo experiments

- Rats (*Rattus norvegicus*, WISTAR) treated with 1 mg/kg of siRNA (0.33mg/rat)
- Three rats per siRNA, control group treated with water
- Treatment by a single *i.v.* administration
- Sample collection of urine and plasma after 4, 9, 24, 33 and 48 hours
- Free access to food and water

Workflow

100-200 µL of urine (DEPC treated)

add ethanol

load sample column 1

add ethanol

load sample column 2

wash

elute with water

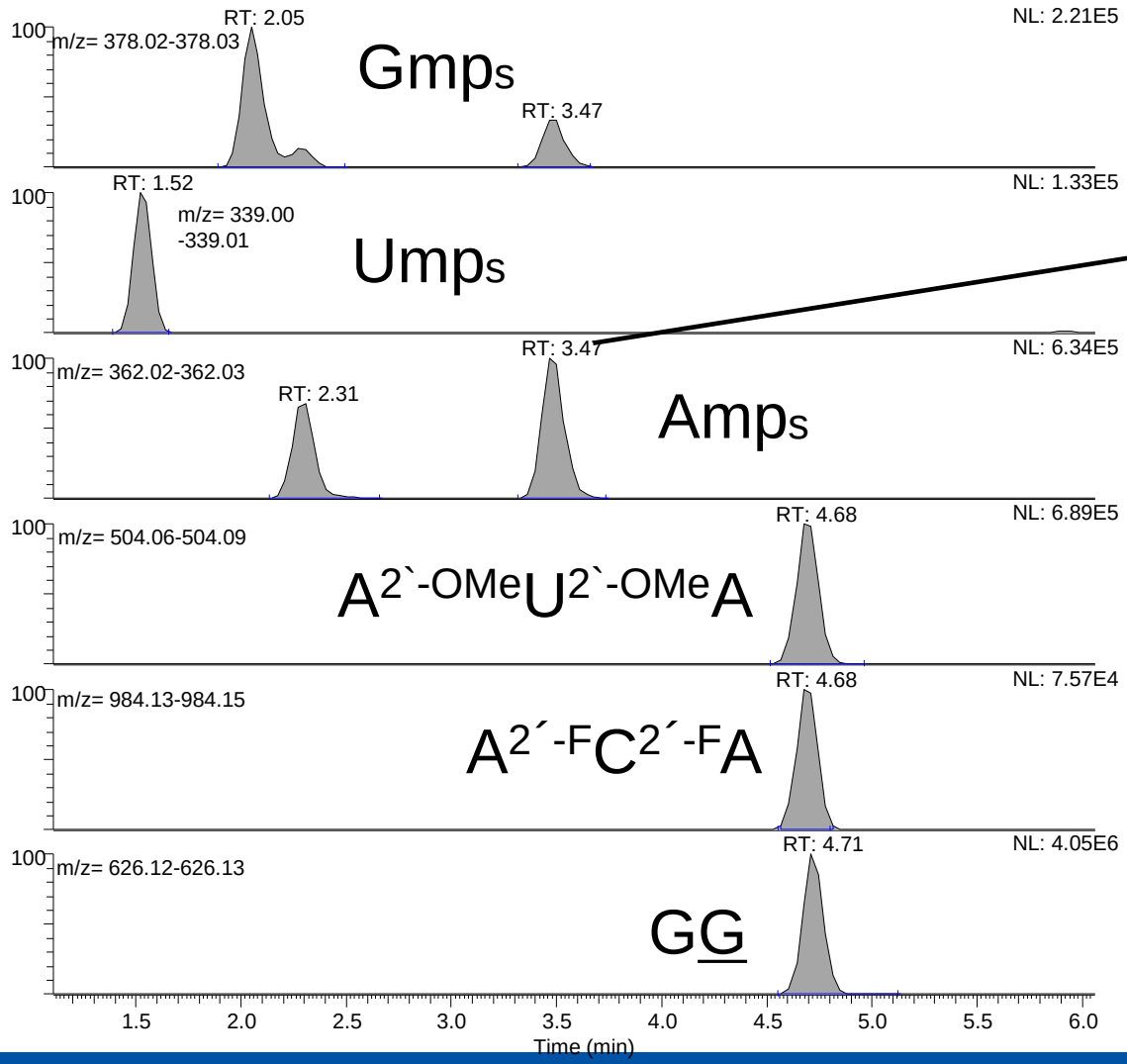
miRNA purification
spin columns from Invitrogen
(Karlsruhe, Germany)

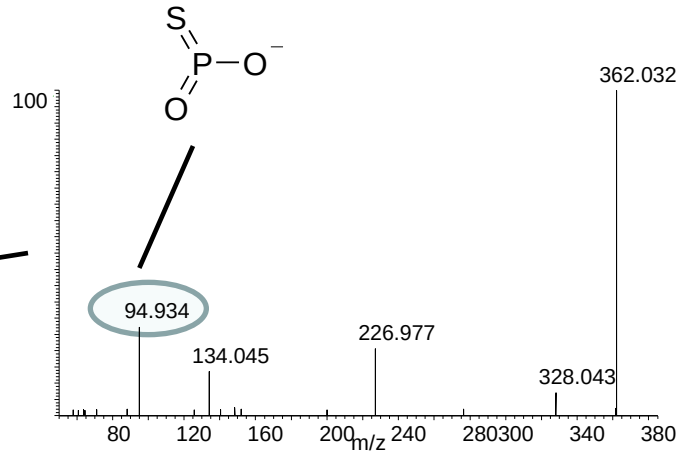
Hydrolysis with 0.1 M NaOH

LC-HRMS

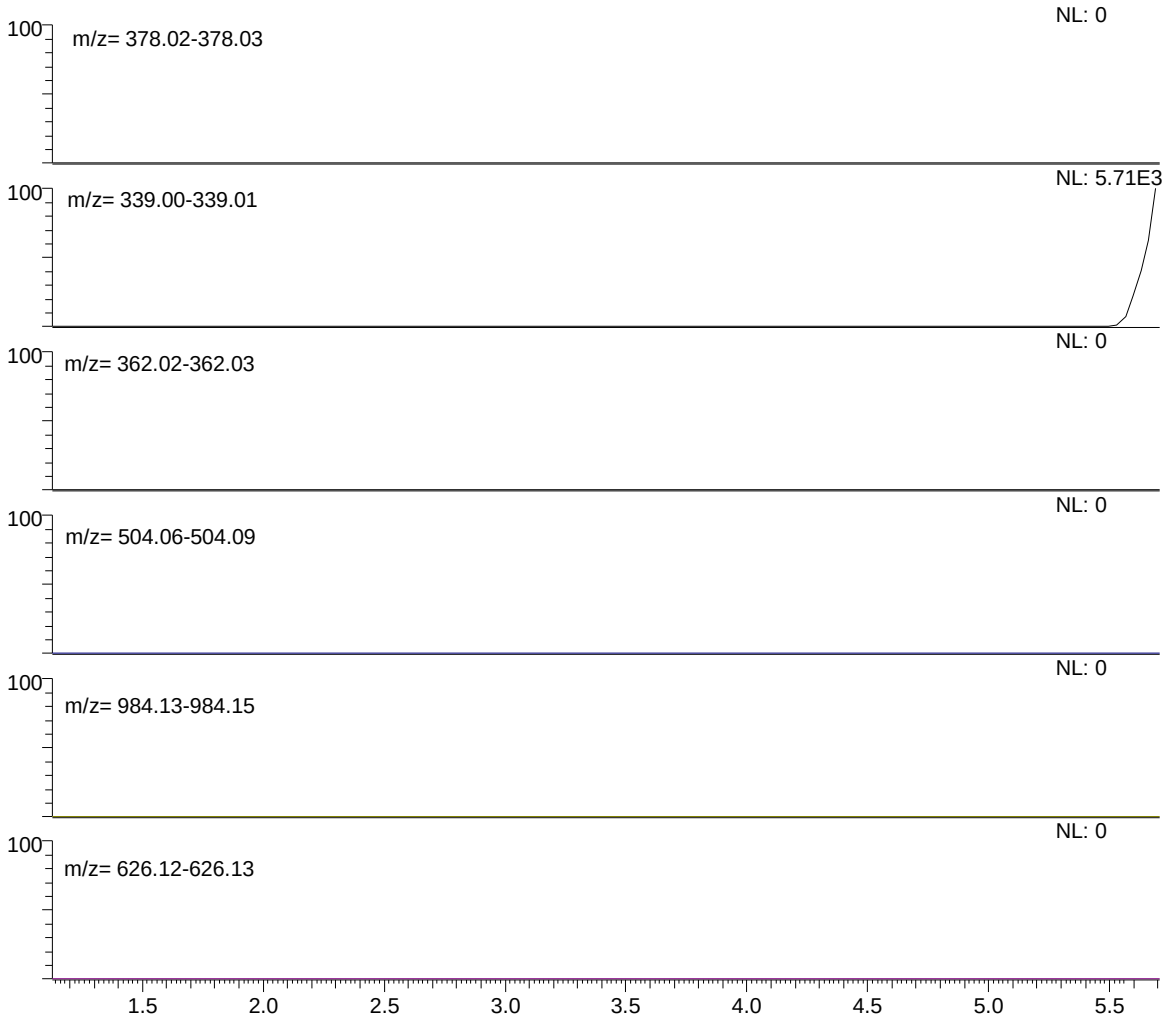
*Kohler M, Thomas A, Walpurgis K, Schänzer W, Thevis M. Anal Bioanal Chem. 2010;398:1305-13012

Hydrolysed rat urine sample (treated with siRNA 1 (4h))





Hydrolysed rat urine sample (control group (4h))



Gmps

Umps

Amps

$A^{2`\text{-OMe}}U^{2`\text{-OMe}}A$

$A^{2´\text{-F}}C^{2´\text{-F}}A$

GG

Workflow

100-200 µL of urine (DEPC treated)

add ethanol

load sample column 1

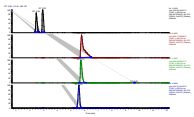
add ethanol

load sample column 2

wash

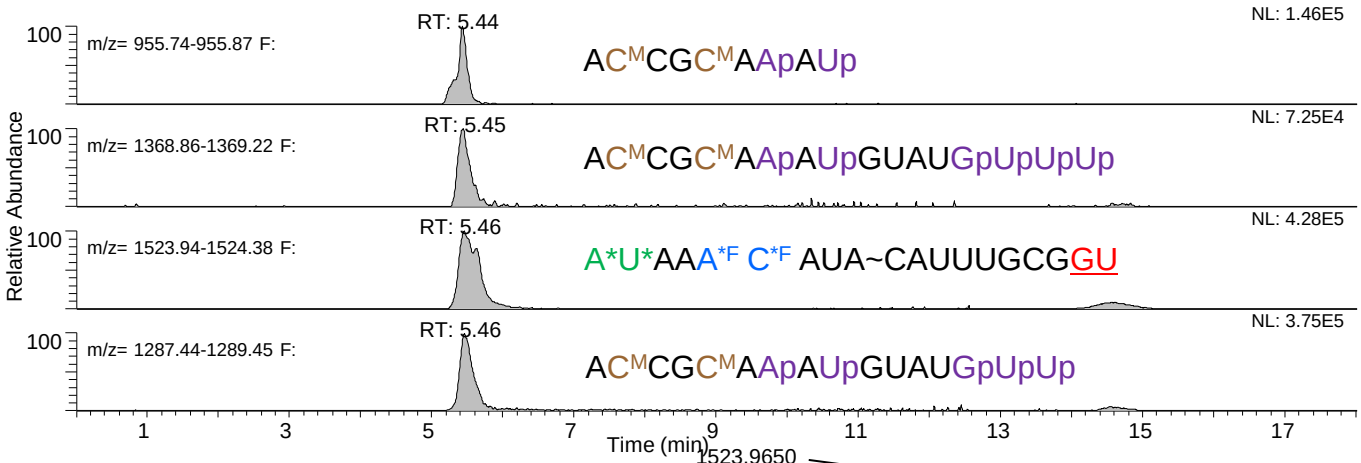
elute with water → LC-HRMS

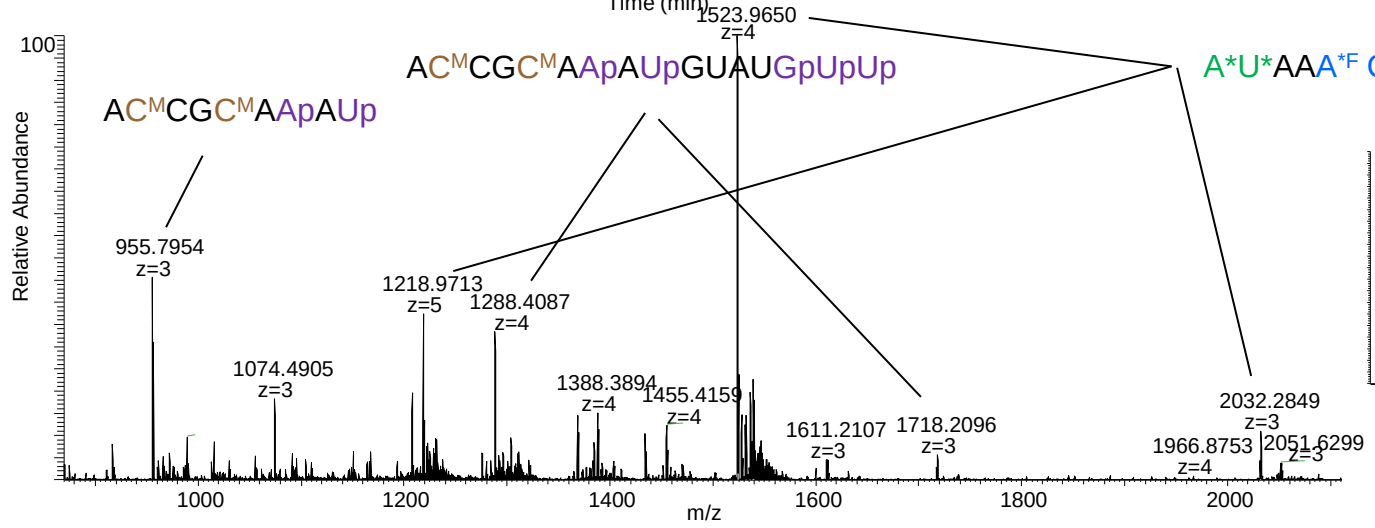
Hydrolysis with 0.1 M NaOH → LC-HRMS

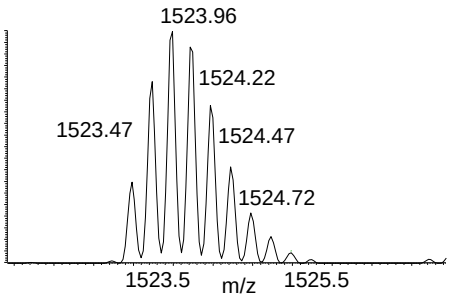


miRNA purification
spin columns from Invitrogen
(Karlsruhe, Germany)

LC-HRMS analysis of intact siRNA from rat urine (treated with siRNA 1 (4h))







Workflow

100-200 µL of urine (DEPC treated)

add ethanol

load sample column 1

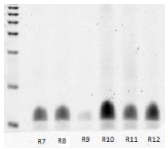
add ethanol

load sample column 2

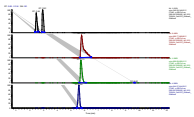
wash

elute with water → LC-HRMS

SDS- PAGE

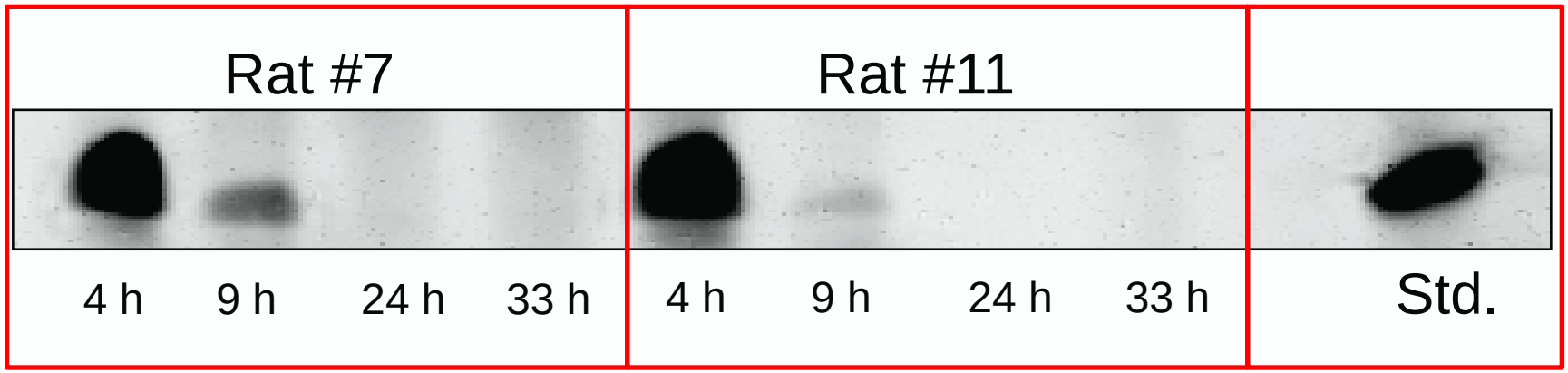


Hydrolysis with 0.1 M NaOH → LC-HRMS



SDS-Page analysis

- Denaturing polyacrylamide TBE-Urea Gels (15%)
- 5 µl of sample + 5 µL of sample buffer
- Heat for 3 min at 70° C
- 180 V const, 75 min
- Stain with SyBr-Safe
- Scan with a Typhoon fluorescence scanner (GE, 488 nm, Filter 520 nm BP 40)



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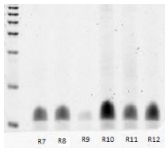
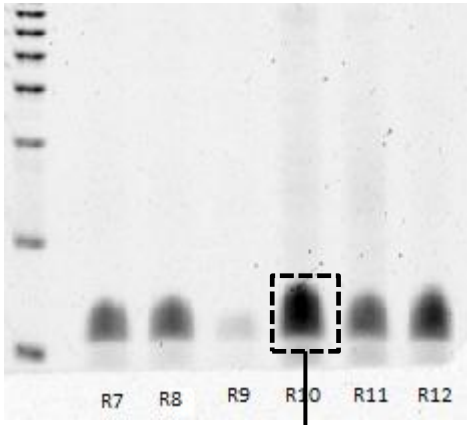
SDS- PAGE

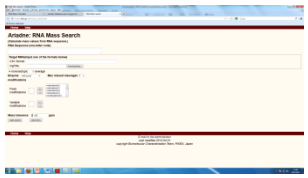




Digest
(RNAse T1, A)

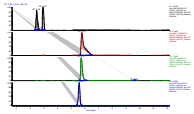
LC-HR-MS/MS

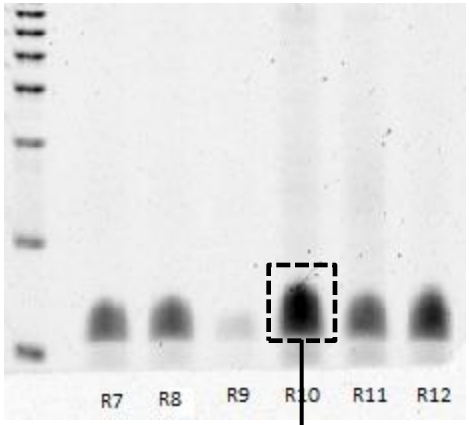
Database
search



Hydrolysis with 0.1 M NaOH → LC-HRMS

LC-HRMS





Digest
(RNAse T1, A)

Experimental „RNomics“

Digestion conditions:

-RNA bands were excised

-Cut into small pieces

-Digested with RNAse (T1 or A) for 1 h at 37° C

-Centrifugation

-Supernatant into fresh tube

LC-HR-MS/MS

MS conditions

-Full scan analysis in negative mode (Res 70 000 FWHM)

-Data dependent MS/MS triggering, if charge state (*m/z)* is < -1

-File converting

Database Search
http://ariadne.riken.jp/index.html

Identification of anti-Myostatin RNA

Not for modified siRNA

Validation results:

	Sense 1	Antisense 1
Specificity	No interfering signals (n = 10)	
Precision (n=6)	20 %	18 %
Recovery (n=6)	18 %	17 %
Limit of detection	~25 pmol/ml of urine	~25 pmol/ml of urine
Linearity	y= 0.3655 x-0.1374, R=0.992	y = 0.1264 x+0.0002, R=0.992

Conclusion

- Modern doping control analytical assays include GC-MS(/MS), LC-MS(/MS), electrophoretic, immunological, and combined approaches

- Comprehensive coverage of doping agents given – loopholes still present

- State-of-the-art equipment allows today detecting the administration of siRNA as one of the prohibited gene doping strategies

- Continuous improvement of analytical methods and their implementation in routine doping controls essential