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Scientific developments and challenges in testing on LC-MS/MS in NDTL, India (2009-2013)

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Abstract

National Dope Testing Laboratory (NDTL), India is engaged in the fight against doping and the anti-doping program in India is strengthened year by year by continuous efforts made by NDTL through its stringent testing protocol, research and education. The combination of high performance liquid chromatography with mass spectrometry (LC-MS/MS) is a boon for detection of WADA banned drugs. The present study extensively reviews the history, routine doping control, adverse analytical findings (AAFs) and research analysis reported by NDTL using LC-MS/MS.
The LC-MS/MS screening procedure was started with 12 compounds (S9) and currently covers most of the categories of drugs from the WADA prohibited list (S0, S1, S3, S4, S5, S6, S7, S9, M1, P2). This multi-analyte target approach has significantly reduced the turn-around-time (TAT), manpower and is cost effective. The review of AAFs reported by NDTL in last five years shows that more than 50% of AAFs were reported using LC-MS/MS.
From 2009-2013, 683 AAFs reported using LC-MS/MS, out of which 8.4% were reported below the WADA MRPL. The use of LC-MS/MS has made contributions in the research activities, new method development; metabolite identification and testing of herbal drugs adulteration in NDTL India. The statistical analysis of AAFs reported shows that the technique has significantly improved the reporting of results with lower limit of detection. The application of LC-MS/MS on doping related peptides like Insulin and Insulin like growth factors is in progress at NDTL, India.

Introduction

The issue of doping in sport is multifaceted. National Dope Testing Laboratory (NDTL), India is engaged in the fight against doping since 1990. The Anti-doping program in India is strengthened by continuous efforts made by the lab through its stringent testing protocol, extensive research and education. The combination of high performance liquid chromatography with mass spectrometry (LC-MS/MS) has made a significant impact in the field of doping analysis. It has undergone tremendous technological improvements allowing detection of various categories of small molecules, peptides and proteins [1-4]. LC-MS/MS has become an integral part of sports drug testing. The present paper elaborates five years of comprehensive experience, developments and challenges of analytical assays employing LC-MS/MS for various classes of drugs of WADA prohibited list [5].

Experimental

The study extensively reviews the history, routine doping control, adverse analytical findings (AAFs) and research analysis reported by NDTL using LC-MS/MS technique. The findings are presented with over all comparisons and statistical significance.

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Results and Discussion

The laboratory is doing routine analysis on LC-MS/MS since 2006 and the initial LC-MS/MS screening method included screening of 12 glucocorticosteroids which developed over a period of time to screening of 165 substances. The analytical assays employing LC-MS/MS technique for screening of various categories of drugs in NDTL are depicted in Table 1.

Category	Initial method of testing		Development on LC-MS/MS
	Sample Preparation	*Analytical technique*	
S1- Anabolic Androgenic Steroids (AAS) and other anabolic agents	Enzymatic Hydrolysis, Liquid Liquid Extraction (LLE), Silylation	GC-MS	Improved LOD on LC-MS/MS for Steroids with large conjugated or cross-conjugated electron systems like Trenbolone, Boldenone and Gestrinone due to good proton affinities. Stanozolol - most misused in Indian sport. Inclusion in LC-MS/MS screen led to increased AAF (Stanozolol) since 2009[7]. Inclusion of Methandienone metabolite 4 (nightwatchman) led to 5 fold increase in percent AAF.
S3- β 2 agonists	Enzymatic Hydrolysis, LLE, Silylation	GC-MS	Quantitative analysis for threshold substances Formoterol [8] and Salbutamol
S4- Hormone and metabolic modulators	Enzymatic Hydrolysis, LLE, Silylation	GC-MS	Significant increase in AAFs after inclusion in LC-MS/MS screen in 2011. Occurrence of Clomiphene parent (below 10 ng/ml) excluding the marker 3-methoxy-4-hydroxy Clomiphene observed in 10 samples in NDTL in 2012-2014. Further study on metabolic profile is in progress
S5- Diuretics and other masking agents	LLE Solid Phase Extraction (SPE), Methylation	HPLC-UV/DAD GC-MS	Improved run time (5 min) on LC-MS/MS screening as against GC-MS method (15 min run time) Improved throughput with testing of 12 samples in an hour against 4 samples per hour (GC-MS). Direct injection method with in-source fragmentation for plasma volume expanders facilitates rapid analysis.
S6- Stimulants	Alkaline LLE	GC-MS GC-NPD	Inclusion of methylhexaneamine in 2010 raised the percent AAF for stimulants [9]. Direct injection analysis method for quantitation of Ephedrines (selective and fast) with 6 min of run time as against 15 min (GCMS).
S7- Narcotics	LLE, Silylation and acetylation	GC-MS GC-NPD	Quantitative analysis for Morphine.
S9- Glucocorticosteroids	---	---	LC-MS/MS has been the screening method since incorporation in the scope
P2- Beta blockers	LLE, acetylation	GC-MS	Run time of 7 min on LC-MS/MS as against 15 min (GC-MS method). Improved throughput with testing of 12 samples in an hour against 4 samples per hour (GC-MS).

Table-1: Details of various prohibited groups screened on LC-MS/MS with significant contribution

The reduced minimum required performance limit (MRPL) (20-80%) and Limit of detection (LOD) guidelines as enforced by WADA from January 2013 necessitated the need to improve the existing analytical method (LC-MS/MS) in our lab. The screening method transfer and validation on UPLC-MS/MS 5500 QTrap using polarity switching allowed a fast and sensitive detection of 165 drugs [6]. The review of Adverse Analytical Finding (AAFs) shows that out of the total AAFs of last five years, more than 50% were reported using LC-MS/MS (Figure 1).

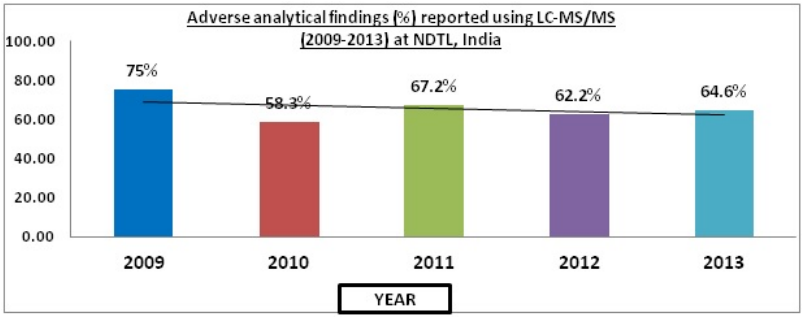

Figure 1: AAFs percentage reported using LC-MS/MS for last five years (2009-2013) at NDTL, India

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Drug wise review of AAFs shows that out of the twenty nine (29) drugs reported as AAF in five years using LC-MS/MS, the top six drugs were Stanozolol (43%), Methylhexaneamine (18.7%), Methandienone (17%), Furosemide (10%), Clenbuterol (7.7%) and d-amphetamine (3.6) (Figure 2).

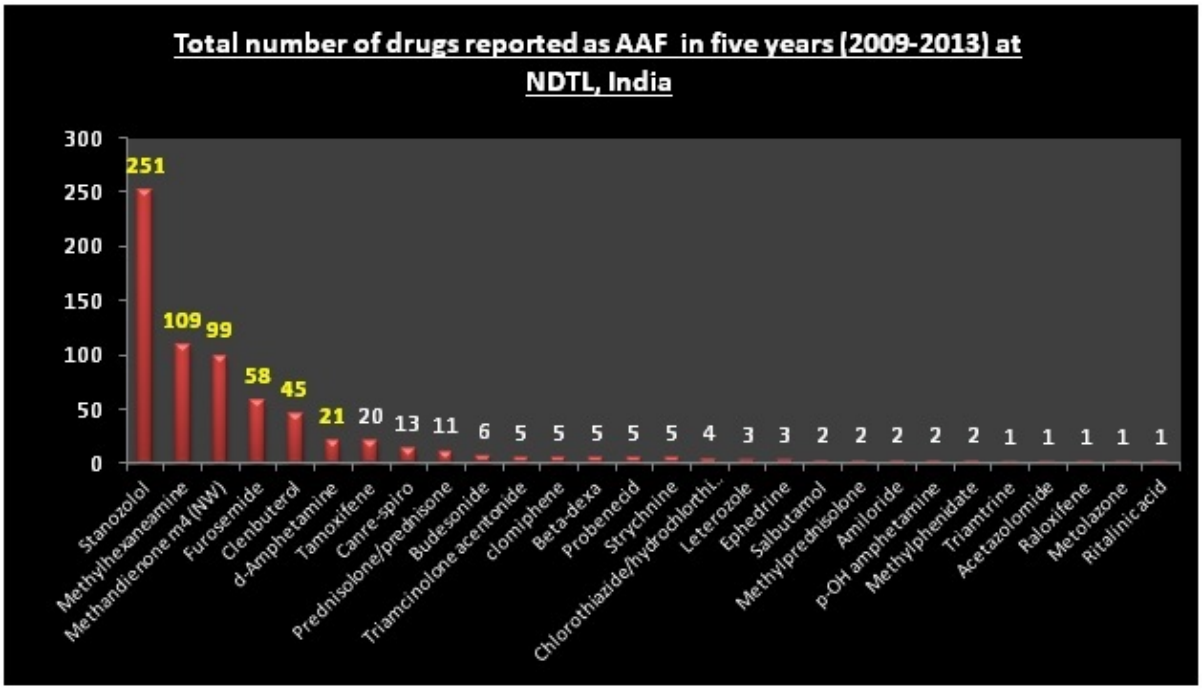

Figure 2: Drug wise AAFs reported using LC-MS/MS technique (2009-2013)

Sudden increase in the AAFs for d-amphetamine was observed in 2013 after its inclusion in the LC-MS/MS screening procedure (Out of 21 cases of AAFs for d-amphetamine, 12 were reported in 2013), which is due to its improved LOD. Similarly, the inclusion of Methandienone metabolite 4 (night watchman) in LC-MS/MS screen resulted in a significant increase in the percent AAF since 2009.
Out of total 889 AAFs reported by NDTL in five years (2009-2013), 683 were reported using LC-MS/MS technique, out of which 8.4% were reported below the requisite WADA MRPL levels. The drug wise bifurcation shows that 100% of findings for Clomiphene and Chlorthiazide were below MRPL followed by Strychnine, Pholedrine and Tamoxifene (Figure3).

Conclusions

LC-MS/MS has significantly influenced the doping control analysis over the past decade. It has revolutionized the analytical processes for small molecules. Numerous drugs with not very good detection on GC-MS are now efficiently included for analysis in LC-MS/MS. Ultra Performance Liquid Chromatography (UPLC) MS/MS has reduced the run time and increased the signal-to-noise ratio of the substances thereby improving sensitivity. The use of LC-MS/MS which started in 2006 in NDTL, India with the testing of glucocorticosteroids and now catering to the screening of approximately 180 compounds from WADA prohibited list. The statistical analysis shows that the improved screening and confirmation methods led to increased AAFs. The application of LC-MS/MS on peptides like Insulin and IGFs is in progress at NDTL.

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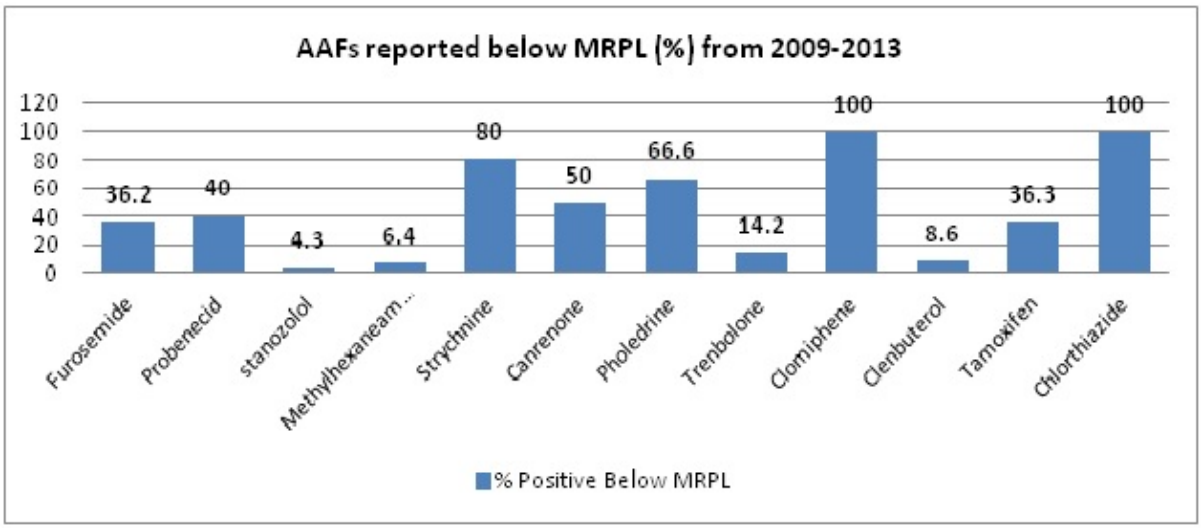

Figure 3: AAFs reported below MRPL in five years (2009-2013) using LC-MS/MS

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