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RECENT ADVANCES IN DOPING ANALYSIS (7)

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Automated Solid Phase Extraction

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Introduction

The sample preparation for anabolic steroids traditionally consists of five steps. These include a solid phase cleanup (XAD2, C18) followed by enzyme hydrolysis, extraction of the steroids, derivatisation and finally GCMS analysis. This process has been used for many years in our laboratory and is based on methodology introduced to us by Prof. M. Donike.

Generally automated procedures for the solid phase cleanup of drugs has served as the means of eliminating the need for a solvent extraction. In order for this to be applied to the steroid screen with a single solid phase process the order of steps would have to change drastically. Thus enzyme hydrolysis has to be performed directly on the urine then solid phase extraction would be used to purify the substances of interest followed by derivatisation and finally GCMS.

Enzyme hydrolysis has been a concern in the past. This is the one step of the process that has always been poorly controlled. There was no easy means of telling if the enzyme has successfully and completely cleaved the glucuronides. If incomplete hydrolysis occurs the detection of anabolic steroids is greatly reduced. This is expected to be even more difficult if performed directly on the urine due to the possibility of the presence of enzyme inhibiting substances. Previously these may have been removed during an initial solid phase cleanup before hydrolysis but again no control was included.

Materials

Internal standards

Deuterated internal standards give us the means of controlling all aspects of the process. A deuterated glucuronide is needed for the hydrolysis control. Internal standards are needed for retention time control and for quantitation. These substances are available to us through a program undertaken by the National Analytical Reference Laboratory (NARL) in Sydney or from commercial sources.

The procedure for monitoring the enzyme hydrolysis was reported by Geyer *et al* (1) and consists of using d5-androsterone glucuronide and d4-etiocholanolone added to the urine in a ratio, which after hydrolysis gives signals for d5-androsterone and d4-etiocholanolone of approximately equal height. We use d4-androsterone glucuronide and d5-etiocholanolone and variations from a ratio of 1 are monitored and an acceptance range can be set.

The use of d3-testosterone and d4-epitestosterone in a ratio of 6:1 allows monitoring of the T/E ratio with greater accuracy. We use d4-epitestosterone because the ion for d3-epitestosterone at m/z 435 interferes with the metabolites of methyltestosterone. Using a low d4-epitestosterone concentration the minor quantity of the d3 substituted material does not interfere with the m/z 435 ion for 17α-methyl-5β-androstane-3α,17β-diol.

Stanozolol has always been a difficult substance to detect. The column and liner conditions need to be closely monitored to ensure detection at low levels is achieved. The d3-3'-hydroxystanozolol metabolite is available from Radian International (Austin Texas, USA) and serves as an excellent means of ensuring the detection of stanozolol metabolites is under control. It also serves as a time marker for this substance making chromatogram reading easier.

Addition of methyltestosterone at the final stage before derivatisation allows it to be used to gauge the recovery of the surrogate internal standards and control the overall process.

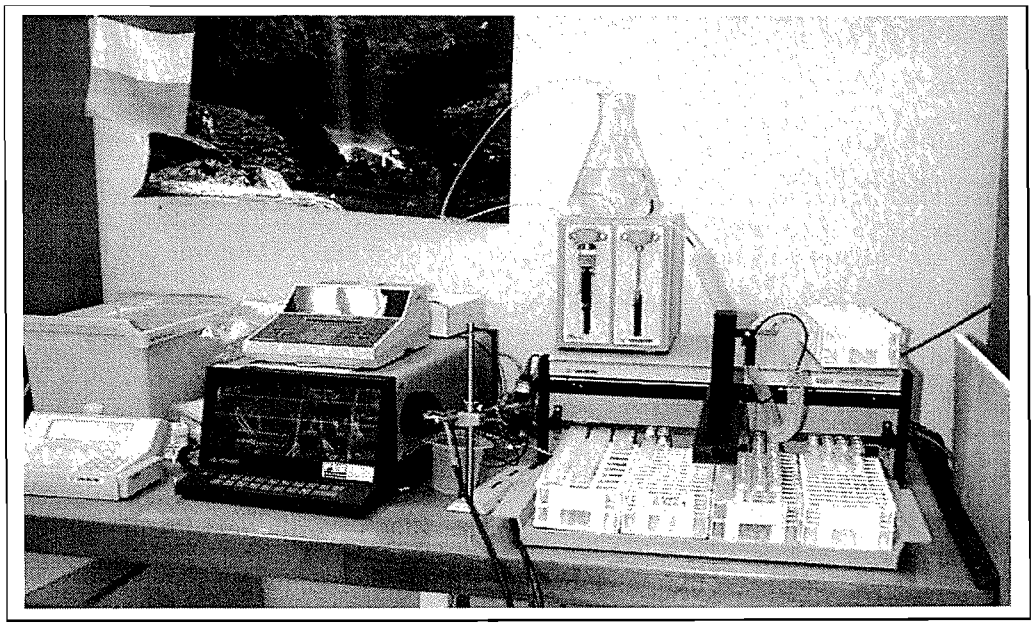
SUBSTANCE	CONC. IN URINE (2mL) ng/mL	TYPE	CONTROL CONDITION	SOURCE OF STANDARD
d3-TESTOSTERONE	100	Surrogate	Recovery >40%	NARL
d4-EPITESTOSTERONE	16.7	Surrogate	d3T/d4E = 6	NARL
d4-ANDROSTERONE GLUCURONIDE	400	Surrogate and hydrolysis control	Ratio heights of d4-androsterone to d5-etiochol anolone >0.6 <1.0	NARL
d5-ETIOCHOLANOLONE	200	surrogate		NARL
d3-3'-HYDROXYSTANOZOLOL	20	surrogate	Peak height and shape acceptable	RADIAN
METHYLTESTOSTERONE	100	Internal standard	Retention time within 10 secs of expected	SIGMA

Methods

A. Sample dispensing

A Gilson XL222 instrument is used for sample dispensing. After sample verification and data entry onto the LIMS system is completed the A sample is opened and a 20mL aliquot is poured into a large test tube. No item is allowed to come into contact with the original sample, which is then stored in a freezer. The test tube is placed into a rack, which has been designed to fit onto the Gilson rack bed. This rack takes 24 tubes. Two other racks containing labeled test tubes (1cm x 5cm) (5 for full screen and 2 for out of competition screen) are placed onto the rack bed to receive the aliquots. The Instrument is programmed to dispense from the sample tube into the receiving tubes in volumes corresponding to requirements for the screens to be performed. The aliquots are then passed to the extraction chemists.

The use of this instrument allows unattended dispensing as well as limiting the handling of the urine giving better Occupational Health and Safety conditions. It has also been programmed to automatically measure pH (flow cell) and specific gravity.



Gilson XL222

B. Sample extraction

The Gilson ASPEC XL4 is used for sample preparation. It has considerable programming versatility, allows up to 5 elution solvents and operates on 4 samples simultaneously. The solid phase programme takes 20 mins for 4 samples and can take tubes and cartridges for up to 80 samples in a batch.

The solid phase extraction (SPE) cartridge was the Varian 3M Empore C18 Disk. It gave good recovery across a very wide range of metabolites. As it is a disk of low bed volume the elution volumes are small thus saving on solvents and evaporation time. However, it suffers from a high backpressure problem especially if the urine has heavy particulates present. By using very slow sample introduction and elution this problem can be overcome. A packed cartridge version of this column is used if manual SPE is performed.

The procedure is given below:

- Take 2mL urine add surrogate internal standards
- Buffer urine to pH 7, add E. coli glucuronidase, and incubate at 50°C for 1.5 hours.
- Set up Gilson ASPEC XL4 with solvents and cartridges.
- Wash needles and prime solvents
- Condition cartridges with 2mL methanol then with 2mL water
- Load hydrolysed sample onto cartridges (slowly)
- Wash with 1mL water then 0.5mL 25% methanol/water
- Dry cartridge with nitrogen for 5 mins.
- Wash cartridge with 1mL hexane then dry with nitrogen
- Elute over collection rack with 2 x 1mL ethyl acetate containing 5% methanol (slowly)
- Add methyltestosterone internal standard to collection rack

Results

The SPE method was validated against the XAD-2/hydrolysis/ether extraction procedure used in most IOC laboratories by analysis of multiple runs of a known multi drug mixture prepared by combining excretion studies of several steroids. The between run variation in signal was measured by comparison of the analyte signal height to that of the surrogate d3-Testosterone. The table below shows the CV% for the runs n=7. This shows the considerable day to day variation for the analytes.

BETWEEN RUN VARIATION USING EXCRETION MIX

SUBSTANCE	MEAN (ANALYTE/d3T)	% CV
T/E	0.87	3.4
d3-TESTOSTERONE	1	
NORTESTOSTERONE M1	0.46	13.7
DROSTANOLONE M	1.9	25.9
METENOLONE M	0.09	37.7
BOLDENONE M	1.36	5.9
CLOSTEBOL M	0.24	15.8
MESTEROLONE M	1.8	27.2
METHYLTESTOSTERONE M1	0.06	7.4
NORETHANDOLONE M1	0.06	24.8
OXYMESTERONE M	2.72	14.3
OXYMETHOLONE M	1.4	6.2
PROBENECID	0.33	29.7
3'-OH-STANOZOLOL	0.12	16.8
TERBUTALINE	0.47	9.8
SALBUTAMOL	0.58	14.7
CODEINE	0.26	13.3
MORPHINE	0.07	49.6
AMILORIDE	0.05	53.2
TRIAMTERENE	0.25	28.7
CLENBUTEROL	0.11	10.6

The recoveries of the drugs relative to the XAD-2 extraction procedure previously used were determined by analysing the same multi component mixture by both methods. In order to move to the new method all analytes had to give comparable or better performance with the Gilson method.

RECOVERY RELATIVE TO PAD2 METHOD

COMPOUND	RELATIVE RECOVERY (TO XAD2 METHOD) ADJUSTED BY METHYLTESTOSERONE
NORETHANDROLONE M	110
NORPREGNANTRIOL	123
OXYMESTERONE M	105
OXYMETHOLONE M	253
d3-3'OH-STANOZOLOL	54 *
3'-OH-STANOZOLOL	110
PROBENECID	1764
CANRENONE	209
DANAZOL M	245
TERBUTALINE	124
SALBUTAMOL	200
CODEINE	78
MORPHINE	192
OXANDROLONE M	103
TRIAMTERENE	218
6β-OH-METHANDIENONE	221
CLENBUTEROL	103
TRENBOLONE ART.	183
d4-ANDROSTERONE	90
d5-ETIOCHOLANOLONE	103
METHYLTESTOSTERONE	100
d3-TESTOSTERONE	112
TESTOSTERONE	122
DHT	97
EPITESTOSTERONE	116
5α-ANDROSTAN-3α,17β-DIOL	110
5β-ANDROSTAN-3α,17β-DIOL	119
5α-ANDROSTAN-3β,17β-DIOL	184
ANDROSTERONE	84
ETIOCHOLANOLONE	112
11β-OH-ANDROSTERONE	74
11β-OH-ETIOCHOLANOLONE	73
DHEA	124
NORTESTOSTERONE M	97
DROSTANOLONE M	58
METHENOLONE M *	295
BOLDENONE M	119
BOLDENONE PC	121
CLOSTEBOL M	86
MESTEROLONE M	64
METHYLTESTOSTERONE M	91

* HAS INTERFERENCE, M = metabolite

Conclusion

The process has been used successfully within our laboratory for over 6 months. For "normal" samples the process rarely fails. Failures are easily detected by monitoring the control conditions and repeating as necessary. Possible enzyme inhibition has rarely been found. We have noticed some samples which have resulted in d4-androsterone/d5-etiocholanolone ratios >1.0. These appear to be samples that have high bacterial content often from wheelchair athletes. The non-conjugated surrogate was more rapidly degraded than steroids present as conjugates in the urine hydrolysis stage. For these samples a fresh aliquot from the thawed A sample is taken. The PAD-2 method is used as solid phase cleanup before deconjugation and the process kills the offending bacteria.

Acknowledgements

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Reference

(1) H. Geyer, W. Schaenzer, U. Mareck-Engelke, E. Nolteernsting, G. Opfermann, Screening Procedures for Anabolic Steroids – The Control of the Hydrolysis with Deuterated Androsterone Glucuronide and Studies with Direct Hydrolysis, Recent Advances in Doping Control (5), Proceedings of the Manfred Donike Workshop, 15th Cologne Workshop on Dope Analysis 23rd to 28th February 1997, Sport and Buch Strauss, Germany 1998