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Automated Preparation for Screening of Anabolic Steroids In Urine in HP 7686 PrepStation System.

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ABSTRACT

A complete automated sample preparation for screening of anabolic steroids in urine has been developed. The samples were processed in a Hewlett-Packard 7686 SPE PrepStation System. Each aliquot of 0.6 ml of urine was hydrolyzed, extracted with tert-butylmethylether, dried and derivatized with MSTFA in a 2 ml vial without any hands-on labor. It seems that time of hydrolysis, type of shaking, number of extractions and volume of derivatizating agents have an important effect on the performance of the method. Some results of the validation experiments are shown. The automated method allows to achieve the same sensitivity level as the usual manual handling of the sample, while obtaining an important improvement in terms of laboratory productivity, accuracy and minimization of employee exposure to hazardous materials.

INSTRUMENT.

All experiments were performed with the standalone type of SPE PrepStation. It consists of a tower, automated tray and barcode reader/mixer. In the tower there is a standard syringe pump of 2.5 ml, which can dispense the liquids from reservoirs through the needle to the vial. It contains also two needles for aspirating and dispensing the reagents and another for the evaporation and heating of the sample (30-90°C). The extraction is achieved in the rotatory mixer by repeating the cycle of ¨mix¨ and ¨stop¨.

OPTIMIZATION OF THE METHOD

Hydrolysis. Most of the steroids are excreted as glucuronide/sulphate conjugated requiring an enzymatic hydrolysis at 55°C (optimal operation temperature of the enzyme).The

time of hydrolysis for complete deconjugation of endogenous androgenic steroids like cis androsterone and testosterone is shown in fig 1. The deconjugation of testosterone is completed within 8 min., however for cis androsterone 20 min. are needed. Hence a hydrolysis time of 25 minutes. at 55°C was selected for deconjugation of the anabolic agents studied here.

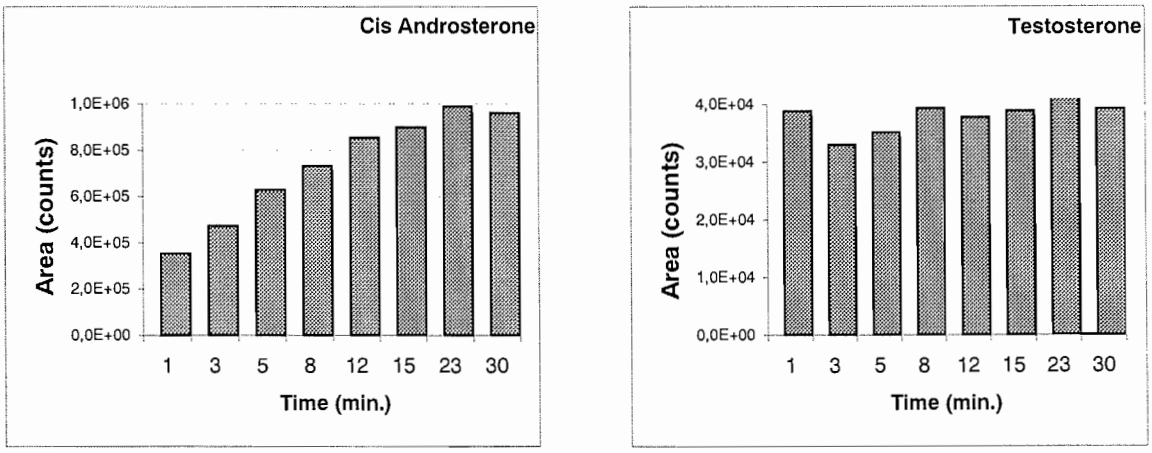

Fig.1 Influence of time on hydrolysis.

Shaking. The effect of different ways of shaking seems to play an important role in the method performance. Due to the limited free space in the vial for mixing of two phases it was observed that the number of " breaks " during the extraction was important in the extraction process. A 20-steps mixing was used in the final method.

Multiextraction. The improvement in recovery of some endogenous androgenic steroids when multiple tert-butylmethylether extractions were carried out is shown in Fig.2. An increase of 20% in the recovery of most compounds was observed after a second extraction. After a third extraction the values increase only with 5%. As each new extraction involves a time of preparation of 20 minutes and taking into account the improvement in terms of recovery a double extraction will be used in this method.

RESULTS

Carry over. Table I shows the result of the carry over study. The effect of processing samples with really high concentrations of anabolic agents was evaluated with a batch of 12

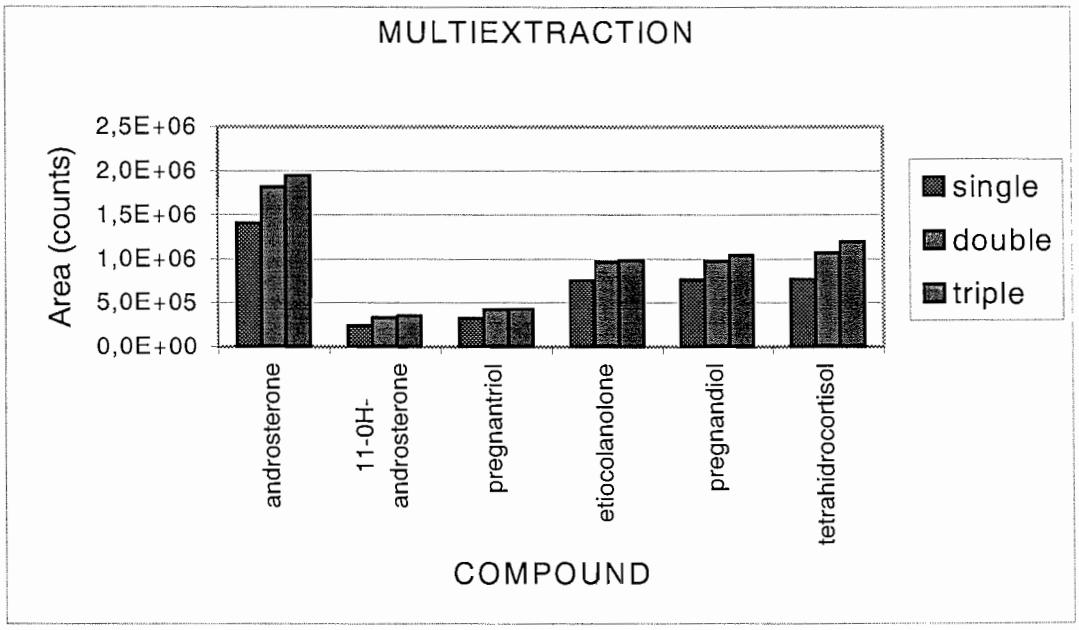

Fig.2 Multiextraction

samples. Between each fortified two negative urines were processed. 19-Norandrosterone was used for spiking , concentration range was 125-1000 ng/ml. No carry over was observed.

Tab.1 Carry over.

Sample N° in Queue	Target Fortified Concentration (ng/ml)	Assayed Concentration (ng/ml)
1	125	115
2	Blank	0
3	Blank	0
4	250	238
5	Blank	0
6	Blank	0
7	500	489
8	Blank	0
9	Blank	0
10	1000	830
11	Blank	0
12	Blank	0

Parallel study. The aim of this study was to compare the efficiency of the manual sample processing and the automatic preparation with the PrepStation.

Table II shows the results for the recoveries. The recovery obtained after the automated extraction can be considered useful for screening purposes.

Tab.II

ENDOGENOUS ANABOLIC STEROIDS

COMPOUND	MAN		PREP	
	con ISTD	STDVE	con ISTD	STDVE
cis-Androsterone	84%	8%	81%	5%
Etiocolanolone	90%	4%	87%	6%
5aAndrostandiol	91%	6%	109%	13%
5bAndrostandiol	80%	8%	89%	7%
Dehydroepiandrosterone (DHEA)	90%	4%	87%	6%
Epitestosterone	95%	7%	86%	7%
Dihydrotestosterone	92%	4%	83%	6%
Testosterone	93%	6%	84%	8%
11-OH-Androsterone	80%	10%	79%	8%
11-OH-Etiocholanolone	88%	2%	85%	7%
Pregnandiol	81%	4%	80%	6%
Pregnantriol	56%	14%	78%	7%
Tetrahydrocortisol	103%	13%	59%	6%

EXOGENOUS ANABOLIC STEROIDS

Metenolona M1 (Ion446)	81%	10%	68%	10%
Mesterolona M1 (Ion 448)	70%	5%	45%	18%
19-Norandrosterone (Ion 405)	82%	9%	76%	6%
19- Noretiocholanolone (Ion 405)	81%	17%	78%	7%
Epimetendiol (Ion 358)	77%	8%	65%	8%
Bolast/Caluste PC (Ion 445)	89%	6%	81%	7%
Bolast/Caluste M1 (Ion 284)	79%	8%	68%	7%
Drostanolona (Ion 433)	81%	8%	66%	8%
Metiltestosterona (Ion 435)	76%	8%	67%	9%
Boldenona M1 (Ion 194)	88%	21%	76%	6%
Noretandrolona M2 (Ion 421)	82%	9%	73%	6%
Clortestosterona M1(Ion 466)	84%	10%	79%	5%
Furazabol PC (Ion 387)	85%	17,0%	77%	5%
3-OH Estanozolol (Ion 560)	63%	11%	35%	2%
Formebolona (Ion 205)	70%	16%	89%	12%
Oximesterona PC (Ion 534)	80%	25%	76%	32%

Productivity and costs. Tables III, IV and V shows in details the quantities of reagents, solvents and time used in step for one sample preparation. In the automated sample preparation there is a saving of reagents what is conditioned by a smaller volume of the sample

and the vial capacity. On the other hand it is necessary to wash the flow paths of the PrepStation and additional costs occur, which don't have place in the manual preparation. Considering the time of one sample preparation the PrepStation wins, but the manual preparation will start to have advantage when bigger batches of samples are processed.
In the automated method the calculated time for 10-sample batch is 12 hours, while in manual processing is about 3 hours. So big increase in time of automated sample preparation is caused by only one position for extraction in the rotatory mixer.

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TAB.III – Reagents.

	PREPSTATION	MANUAL
urine	0,6 ml	2 ml
ISTD	15 ul	50 ul
β-glucuronidase	15 ul	25 ul
pH 7	40 ul	100 ul
pH 11	100 ul	300 ul
MSTFA	20 ul	50 ul
TBME	1 ml	5 ml

TAB. IV – Rinses of the flow paths in PrepStation.

	PREPSTATION	MANUAL
Water	5 ml	0
Methanol	5 ml	0
TBME	8 ml	0
CYCLOHEXANE	5 ml	0
ACETONITRILE	2,5 ml	0

TAB. V - Time of the single sample preparation.

	PREPSTATION	MANUAL
preparation of hydrolysis	13 min	5 min
hydrolysis 55 C	25 min	1 h
change of pH and rinsing (in PREPSTATION)	5 min	8 min
adding TBME and agitation	(18 min)*2	
centrifugation	0 min	5 min
freezing of the water phase	0 min	5 min
drying	(3 min)*2	30 min
derivatization	13 min	30 min
other steps in sample prep.	5 min	5 min**
TOTAL TIME	1h 43 min	2 h 30 min

*preparation of the vials with reagents-PREPSTATION

transfer of the derivatized extracts from tubes into the glass tips in the vials-MANUAL preparation

**increase with the number of samples