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Results of several (small) research projects at DoCoLab in 2005

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1. Introduction

The paper contains a summary of several research projects that were initiated 2005 at DoCoLab and which deal with quality assurance, improvements in detection methods and analysis of new prohormones.

2. Stability studies in urine

Recently, papers on the stability of some doping agents in urine, including caffeine (1), salbutamol (2), cannabis (3), morphine (4), EPO (5) as well as several steroids (6, 7) have been published. In these studies the urine is traditionally sterilised, stabilised and/or filtered. In the current study, untreated urine was spiked with ephedrine, cathine, methylephedrine, 19-norandrosterone glucuronide and a range of diuretics (Table 1).

Long term stability was determined by storage for 1, 2, 3, 6 and 8 months at -20°C, 4°C, room temperature and 37°C. Short term stability was evaluated as the influence of 6 freeze-thaw cycles and 15 h storage at 60°C (simulating two hot days in a car trunk).

For the statistical evaluation of the results for threshold substances, the influence of storage time was based on comparing the measured with the initial concentration taking into account expanded measurement uncertainty (k=2). For comparison of the influence of storage conditions after each storage time, ANOVA-statistics (post hoc Tukey HSD test, α=0,05) were used. For the evaluation of short term stability testing a students t-test (α=0,05) was used.

For diuretics, the evaluation was qualitative and based upon 6 repetitions after each storage condition and time (was the diuretic detected or not?).

The results indicate that ephedrine, methylephedrine and cathine are stable when stored at -20°C and 4°C for a period of up to 8 months. Moreover, only after 8 months storage of urine containing cathine a difference between stable storage conditions (-20°C and 4°C) compared to room temperature and 37°C was observed.

For norandrosterone, the current results largely confirmed the previous findings by van der Merwe and Grobbelaar (6) and show long term stability at 4°C and -20°C during 8 months. However, significant differences were observed when stored at higher temperatures for certain urine samples. The differences largely depended on the urine itself, indicating a possible microbiological cause for degradation. Hence, these results also indicate that previous studies using pretreated urine (filtration, stabilisation, sterilisation) have limited value.

Similar results were found for the testosterone to epitestosterone ratio, which was stable for 8 months at 4°C and -20°C, but where depending on the urinary matrix, already after 3 months degradation effects at higher temperatures were observed.

No degradation effects were observed during the short term stability tests for the threshold substances (both 6 freeze-thaw cycles as well as 15 h storage at 60°C).

For several diuretics, significant degradation effects could be observed already after one month storage at 4°C and higher (Table 1). After 8 months storage at -20°C, no degradation was observed. Moreover, 15 h storage at 60°C also lead to marked degradation phenomena for certain diuretics (Table 2). Short term stability tests showed that up to six freeze-thaw cycles had no effect on any of the investigated substances.

Hence, it is clear that all of the investigated substances are stable in urine during long term storage at -20°C, as done by doping control laboratories. Cooled transportation during hot periods is recommended as a precaution for several heat sensitive diuretics.

Table 1. Qualitative evaluation (number of samples where the diuretic agent was detected in 6 samples) after one month storage at different tempefratures.

	-20°C	**4°C**	**RT**	**37°C**
Mebuthiazide	6/6	0/6	0/6	0/6
Althiazide	6/6	0/6	0/6	0/6
Cyclopenthiazide	6/6	3/6	0/6	0/6
Trichlormethiazide	6/6	5/6	0/6	0/6
Bendroflumethiazide	6/6	6/6	0/6	0/6
Acetazolamide	6/6	6/6	6/6	0/6
Hydrochoorthiazide	6/6	6/6	6/6	0/6
Amiloride	6/6	6/6	6/6	0/6
Etachrynic acid	6/6	6/6	6/6	0/6
Hydroflumethiazide	6/6	6/6	6/6	0/6
Polythiazide	6/6	6/6	6/6	0/6
Bemithiazide	6/6	6/6	6/6	0/6
Epithiazide	6/6	6/6	6/6	0/6
Triamterene	6/6	6/6	6/6	6/6
Diclofenamide	6/6	6/6	6/6	6/6
Chlorthalidone	6/6	6/6	6/6	6/6
Clopamide	6/6	6/6	6/6	6/6
Furosemide	6/6	6/6	6/6	6/6
Torasemide	6/6	6/6	6/6	6/6
Indapamide	6/6	6/6	6/6	6/6
Piretanide	6/6	6/6	6/6	6/6
Xipamide	6/6	6/6	6/6	6/6
Bumetanide	6/6	6/6	6/6	6/6
Probenecid	6/6	6/6	6/6	6/6
Canrenone	6/6	6/6	6/6	6/6

Table 2. Qualitative evaluation (number of samples where the diuretic agent was detected in 6 samples) of the monitored diuretics after 15 h storage at 60°C

substance	SCORE
Althiazide	0/6
Cyclopenthiazide	0/6
Epithiazide	0/6
Mebuthiazide	0/6
Trichlormethiazide	0/6
Acetazolamide	6/6
Amiloride	6/6
Bemithiazide	6/6
Bendroflumethiazide	6/6
Bumetanide	6/6
Canrenone/spironolactone	6/6
Chlorthalidone	6/6
Clopamide	6/6
Diclofenamide	6/6
Etachrynic acid	6/6
Furosemide	6/6
Hydrochoorthiazide	6/6
Hydroflumethiazide	6/6
Indapamide	6/6
Piretanide	6/6
Polythiazide	6/6
Probenecid	6/6
Torasemide	6/6
Triamterene	6/6
Xipamide	6/6

3. GC-MS analysis of anabolic steroids

The routinely used GC-MS method for screening of anabolic steroids (traditionally called extraction procedure IV in doping control laboratories) was revised to include new anabolic steroids, e.g. 2α,17α-dimethyldihydrotestosterone and several other anabolic agents (e.g. zilpaterol). The method is now capable of detecting 72 substances.

Besides adjusting several instrumental parameters (GC temperature program and selected m/z-values for the SIM-method), the extraction recoveries in urine of 57 substances were evaluated with n-pentane, diethylether and ethyl acetate at 2x MRPL-level. These experiments confirmed the previous findings on a selected group of steroids (8) that n-pentane is not suitable as an extraction solvent for screening purposes due to extremely low recoveries of several steroids (e.g. 3'OH-stanozolol, 6β-OH-4-chlorodehydromethyltestosterone, 9α-fluoro-17α-methyl-androst-4-ene-3α,6β,11β,17β-tetrol, …).

Comparison between diethylether and ethyl acetate showed no significant differences in extraction recoveries for the investigated steroids. However, for several of the non-steroidal substances included in the screening procedure, marked differences were observed (Table 3). From these experiments it is clear that ethyl acetate might be used as a valuable alternative to diethylether in extraction procedure IV.

Table 3. Extraction recoveries for several non-steroidal substances with diethylether and ethyl acetate

Substance	Recovery diethylether (%)	Recovery ethyl acetate(%)
Probenecid	5.4 ± 0.9	65.8 ± 5.5
Salbutamol	6.8 ± 2.0	38.2 ± 5.6
Pemoline	49.0 ± 2.0	81.6 ± 6.0
Zilpaterol	19.2 ± 7.2	68.9 ± 8.9
Amiloride	2.4 ± 0.7	36.0 ± 3.4
Triamterene	23.7 ± 4.8	75.4 ± 9.0
Carphedone	14.0 ± 2.9	74.1 ± 8.0
Terbutaline	6.2 ± 2.2	32.7 ± 9.6
Salmeterol	39.0 ± 5.3	78.2 ± 31.1

4. Analysis of new supplements containing prohormones

In 2005, several new designer steroids appeared on the prohormones market and were analyzed by GC/MS and LC/MS.

2α,17α-dimethyldihydrotestosterone was first synthesised in the 1950's by Synthex laboratories and limited data on biological activity has been published (9, 10). It has been reported that unesterified 2α,17α-dimethyldihydrotestosterone is a potent orally active anabolic agent exhibiting only relatively weak androgenic activity. It is sold under the names superdrol, methylmasteron, methasteron and methyldrol.
The full scan TMS-enol-TMS-ether derivatised mass spectrum of this substance is shown in Fig. 1.This steroid is largely excreted as a parent compound and hence doping laboratories should include this substance in their screening procedures.

4-chloro-17α-methyl-androst-4-ene-3 ζ,17β-diol is another new steroid. It is sold under the trade name Promagnon. The full scan TMS-derivatised mass spectrum is shown in Fig. 2. The

fragmentation pathway is fairly simple; showing cleavage of the chlorine atom, as well as loss of two hydroxy-TMS groups. The fragment at m/z 143, is a typical D-ring fragment for 17-hydroxylated and methylated steroids.

Preliminary excretion studies have shown that this steroid is also excreted largely unchanged, and hence detection of its misuse can be based upon the parent compound.

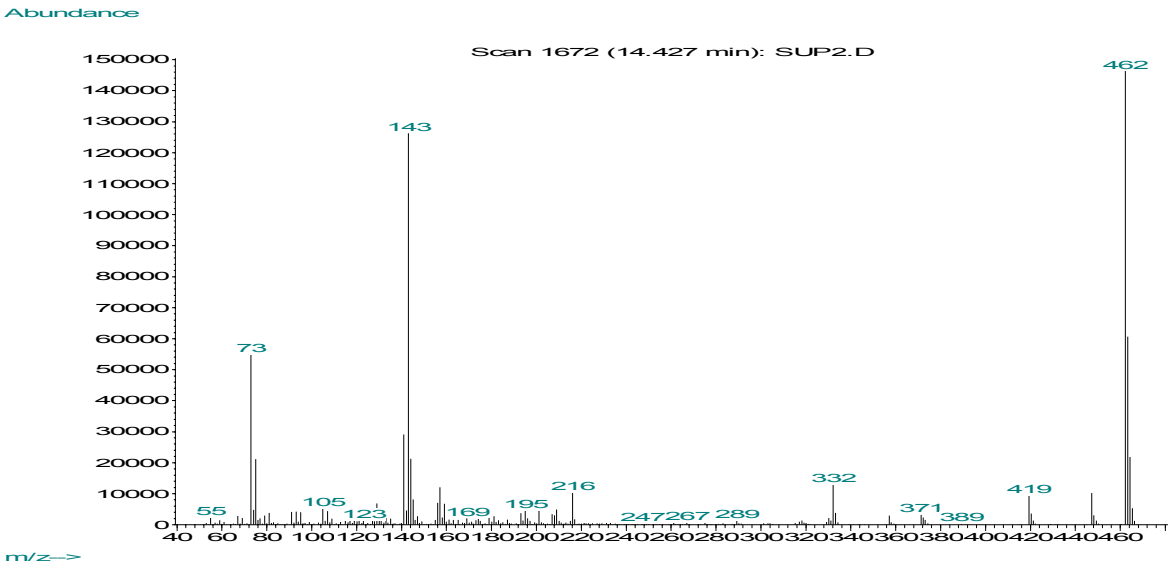

Fig. 1. Full scan mass spectrum of TMS-enol-TMS-ether derivatised 2α,17α-dimethyldihydrotestosterone

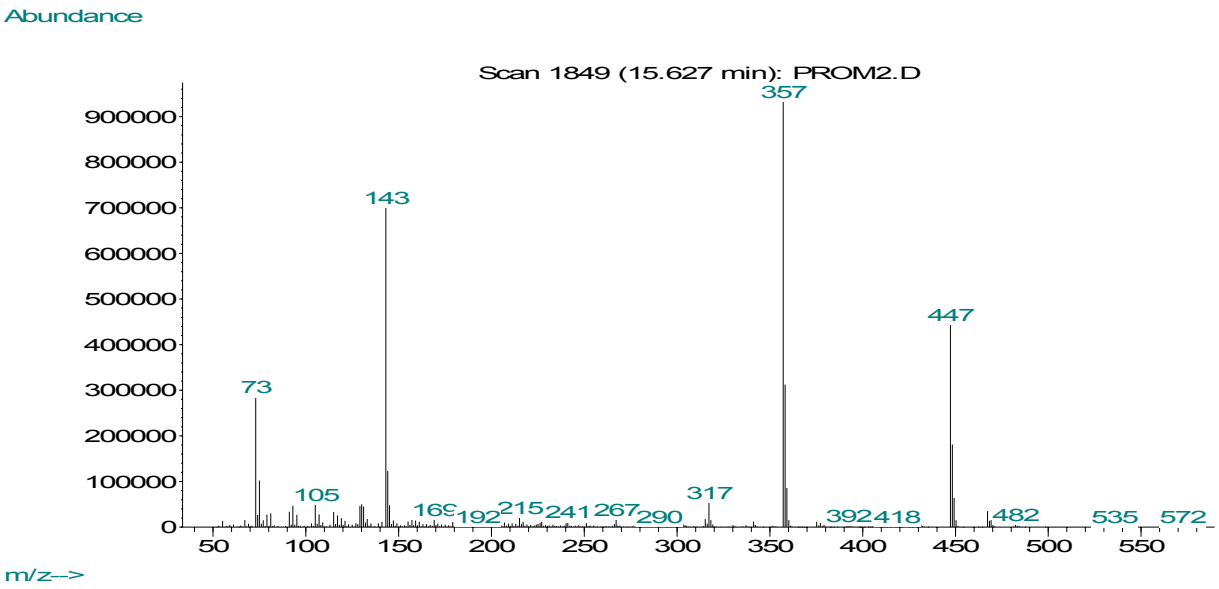

Fig. 2. Full scan mass spectrum of TMS-derivatised 4-chloro-17α-methyl-androst-4-ene-3ζ,17β-diol

5. Acknowledgements

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6. References

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