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Identification problems of urine samples in doping controls resolved by DNA-PCR.

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Abstract

In the last few years, more than 80'000 doping controls have been performed annually worldwide. For all of these urines, sample A and sample B should come from the same individual. However, it happens that appearance and/or analytical results from both A and B samples are not matching, raising the possibility of samples manipulation or miss-labelling.

In Lausanne, we have been recently faced with two samples supposedly originating from the same person, but having very different density values, although similar steroids profiles after correction for creatinine concentrations. Still the question was raised whether or not the two urine samples were really originating from the same individual.

We asked our forensic haemogenetic department to collaborate in this case. It is now common practice in that laboratory to analyse blood or other biological samples (for example : hair) in order to obtain DNA profiles to exclude or to statistically evaluate the inclusion rates between given samples . The new PCR systems (microsatellites and sequence polymorphism) allow the identification of particular samples such as urines which usually contain low amount of DNA as well as PCR inhibitors. Furthermore, analyses of stored urine samples are more problematic because of the degradation of the DNA by microorganisms. In the present case, after statistical evaluation, the probability that both urines were provided by the same person was calculated to be 99.3 %.

Furthermore, balance between the quantity of informations needed and costs should be evaluated before handling the samples.

Introduction

The necessity to identify urine samples arises in different situations. Verification of mixed-up samples in clinical laboratories, drug screening programs and of course doping controls,

where substitution and manipulation of the chain of custody can occur, are the main fields of interest.

Although the analysis of several serological polymorphisms in urine has been already reported in 1965 (1), it is only recently, with new DNA-PCR system typing (Polymerase chain reaction) that a clear identification of urine samples can be performed (2, 3, 4, 5). One difficulty of DNA analyses in urine is the amount of genomic material which is generally very low. If in blood, genomic DNA can be found at a concentration of 40 µg/ml, it is of 20-40 ng/ml in female urine and 2 ng (or less)/ml in male urine.

The yield of specific amplification of human DNA from urine is not only depending on the amount of nucleated epithelial cells and leucocytes in the biological sample, but also on the degree of contamination with bacteria, yeast and fungi. The degree of contamination is influenced by the storage conditions and a considerable difference exists between male and female urines, the latter containing more epithelial cells (3). In general, microscopy of the urinary sediment is recommended as spermatozoa in female urine samples can interfere with the typing of genetic markers (4).

Our laboratory was faced with such a case where A and B urine samples had very different visual aspect (color, sedimentation), specific gravity and pH values, although similar steroid profiles after correction for creatinine concentrations. The question was raised: Are the two urine samples really originating from the same individual. Based on our conventional analytical parameters, it was difficult to state definitively any conclusion. With the help of PCR and DNA typing, it was possible to increase considerably the documentation on the identity of the samples. In order to make these researches on every sample, some recommendations on the storage conditions will be drawn.

Aim of the study

The aim of this study was to compare analytical results from two urines corresponding to A and B samples provided theoretically by the same individual, but presenting suspicious differences in the preanalysis parameters (pH, specific gravity) and absolute concentrations of endogenous compounds.

This comparison was based on two analytical approaches

- a) Steroid profile analysis
- b) Genomic DNA typing using the PCR amplification.

Material and Methods

Sample preparation for steroid profiles

The method of preparation and analysis of the endogenous steroids in urine was similar to what is performed by the Cologne group (6, 7) with some modifications (8).

For sample preparation, the purification of the sample prior to hydrolysis was performed by using 6 ml bakerbond spe (C-18) columns (J.T.Baker, Pilippsburg, NJ, USA) instead of the XAD-2 columns.

The derivatization mixture was MSTFA/TMSI/DTE (1000: 5: 5) instead of MSTFA/NH₄I/ethanethiol and the injection was done on GC-MSD.

GC/MS parameters

GC/MS: HP 5890/HP 5971 (Hewlett Packard)

Column: HP Ultra 1 (OV-1) 17 m, 0.2 mm i.d., 0.33 µm film thickness

carrier gas: 1 ml/min helium at 180°C, splitless

Temperature program: 170°C, 1 min, 1.8°C per min, 229°C, 30 °C per min 310, 5 min.

Preparation and DNA typing

1. Extraction

1-10 ml urine

Sedimentation (10 min, 1500 g)

take 50 µl of the pellet

wash with buffer (Tris, NaCl, EDTA, H₂O)

Centrifuge 14000t / min, 4°C, 5 min

Lysis of histones and membrane
(proteinase K, SDS, DTT, EDTA, NaCl, Tris, H₂O)

Incubation overnight at 37°C

Then extraction with phenol:chloroform 1:1 (2x)

vortex
centrifugation 14000 t/min, 4°C, 5min

Then extraction with chloroform
vortex
centrifugation 14000 t/min, 4°C, 5min

2. Precipitation

add NaCl 5 M and ethanol 100%
mix gently
overnight -20°C

Centrifugation 14000 t/min , 4°C, 20 min
wash the pellet with ethanol
Centrifugation 14000 t/min, 4°C, 5 min

dry under vacuum
dissolved in 20 µl of buffer (Tris, EDTA)
Incubation overnight at 37°C

3. Quantification and Amplification

The Quantity of human DNA was estimated by dot-blot analysis with the Human DNA Quantification System (BRL Life technologies) and 0.5 to 2 ng of human DNA were amplified according to Grange et al (9)

The primers used were those reported by Edward (10) for HUMTH01 and Kimpton (11) for HUMVWF31/A.

4. Detection

Detection was done by native Hydrolink vertical gel electrophoresis in discontinuous buffer followed by silverstaining (9)

Results and Discussion

Physico-chemical parameters and steroid profiles

	A-sample	B-sample
Volume (ml)	40	50
pH	5.3	6.3
Specific gravity	1.024	1.013
Creatinine (mg/ml)	2.5	0.8
Testosterone (1)	154.5	40.9
Testosterone (2)	61.5	50.9
Epitestosterone (1)	16.3	4.3
Epitestosterone (2)	6.5	5.3
Androsterone (1)	3696	1192
Androsterone (2)	1471	1484
Etiocholanolone (1)	3226	955
Etiocholanolone (2)	1284	1190
LH (mIU/ml)	6.9	2.8
T/E	8.3 ± 0.4	8.3 ± 0.2
	(1) expressed in ng/ml	(2) expressed in ng/mg creatinine

Table 1: Physico-chemical parameters and endogenous steroid concentrations and ratios obtained in A and B sample provided theoretically by the same athlete.

The Table 1 gives the values obtained for A- and B- urine samples.

It must be noticed that the pH, specific gravity and creatinine concentration of both samples are quite different and lead to the conclusion that they were provided either at the same time by another person or by the same individual at a different period.

In Switzerland, if it can be proven that B sample is analytically different from A in doping analyses, the final result cannot be validated.

Chromatographic pattern of the A and B samples are quite clearly showing a difference in the abundance of some steroids (Testosterone and Epitestosterone taken as an example in

Figure 1), although this difference is not as spectacular when normalised with creatinine (see Table 1). On the total ion current chromatograms, it can also be observed that the relative abundance of several hydroxysteroids are not similar in the two extracts (see peaks 4, 6 and 7 in Figure 2) which supports the idea that A and B samples are not provided from exactly the same urine.

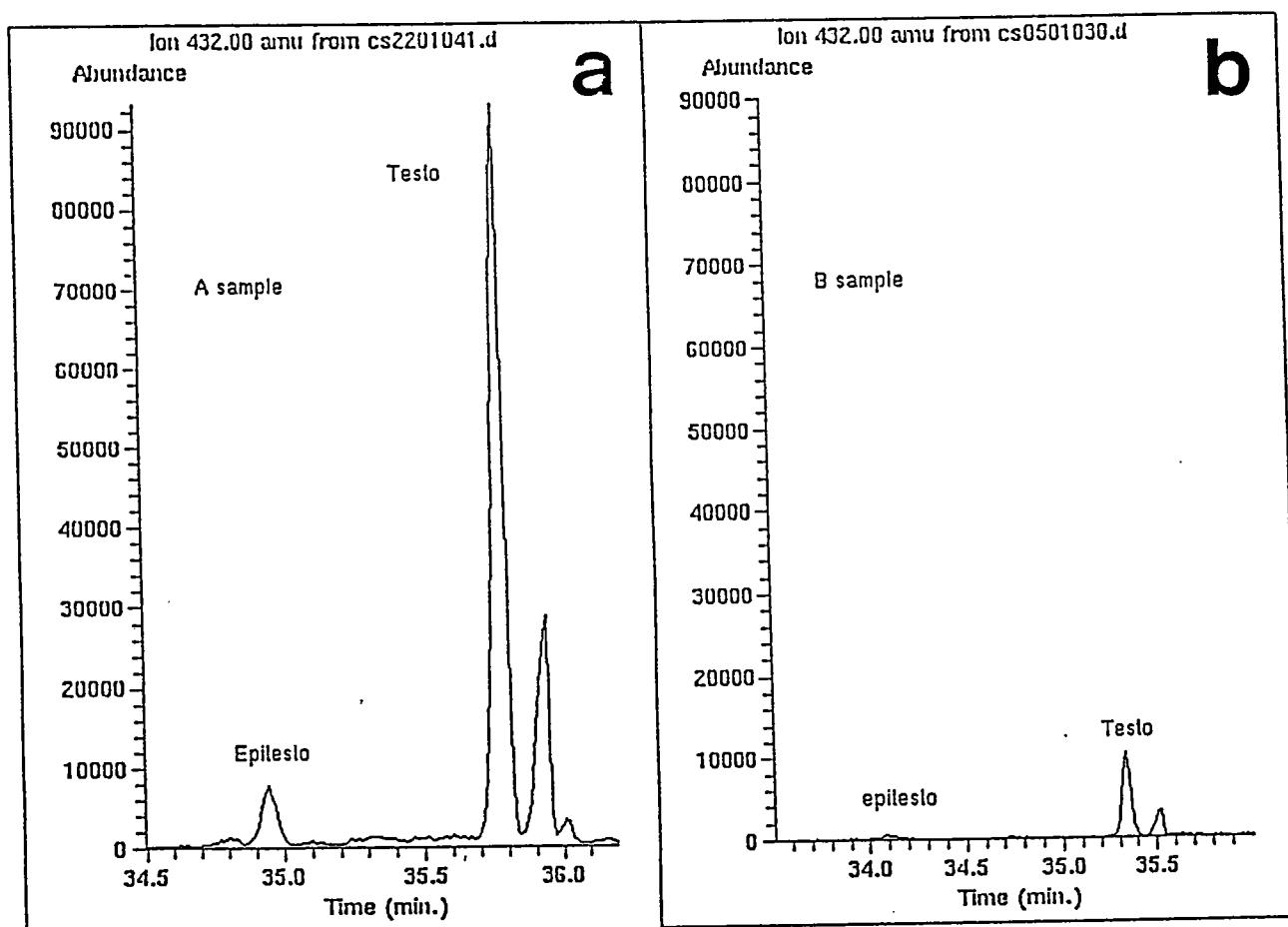


Figure 1: Extracted ion traces $m/z = 432$ from injections in the scan mode of urine sample A (a) and B (b) extract after TMS derivatization. The Y scale was chosen to be the same in order to see the difference in absolute abundance.

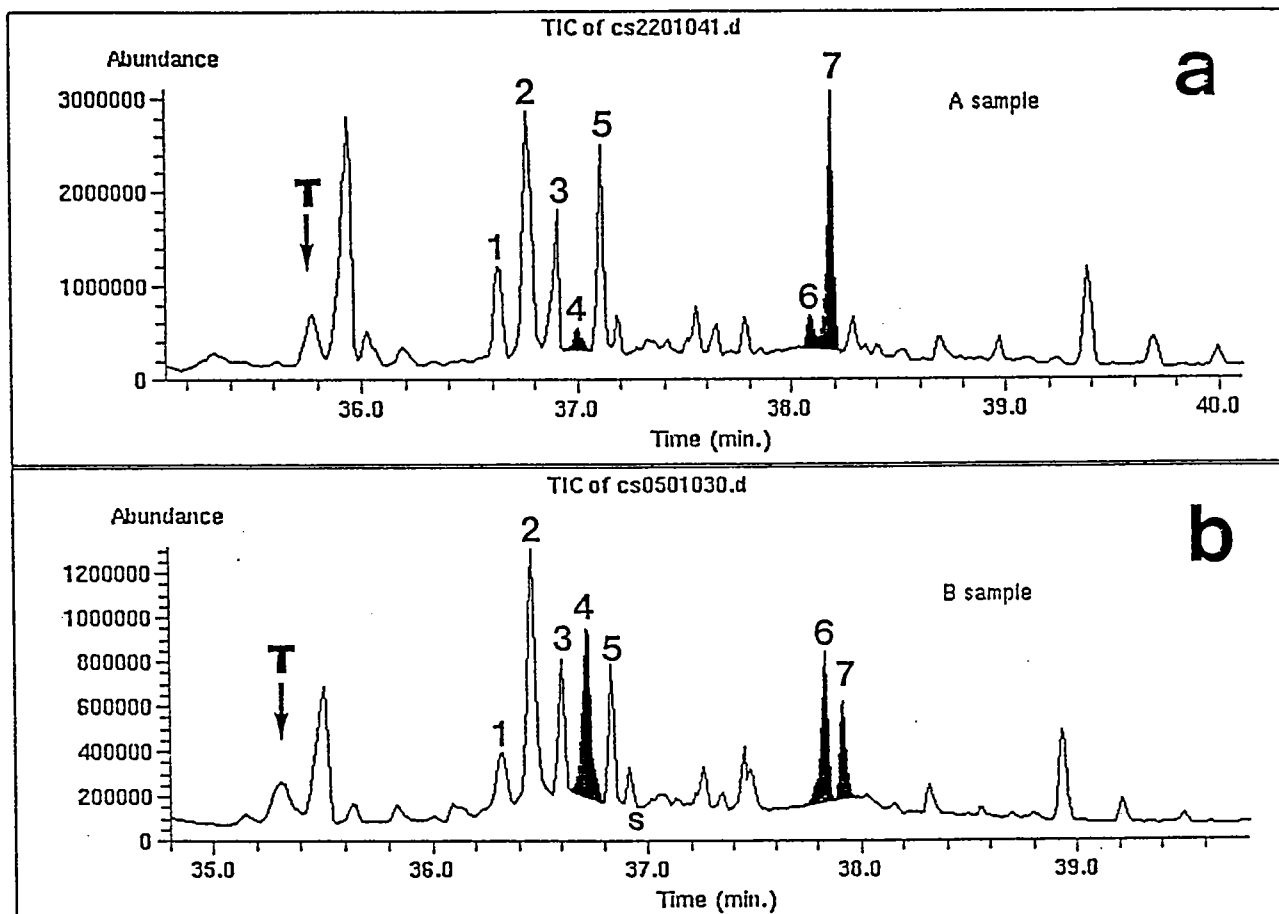


Figure 2: Total ion current chromatograms from injections in the scan mode of urine sample A (a) and B (b) extract after TMS derivatization. T corresponds to the testosterone R.T. and the peaks 1 to 7 are supposed to be hydroxysteroids, but not strictly identified in this case.

Microscopical observations.

Before DNA extraction, the urine is centrifugated in order to use the sediment for PCR analysis.

The microscopical observation of the sediment allows to estimate the degradation of epithelial cells and the occurrence of crystals and fungi or bacterial contamination.

In the present case, the microscopical preparations showed clear differences between A and B samples, mainly in the occurrence of intact nucleated cells (see Table 2)

	A-sample	B-sample
Amorphous cristals	+	+
Oxalate cristals	+++	+
Epithelial cells	-	+
Erythrocytes	-	+
Leucocytes	-	+
Cell fragments	+	+

Table 2: Microscopic evaluation of the sediments observed in A and B sample provided theoretically by the same athlete

PCR investigations

After extraction and amplification of DNA of both samples, hydrolink gel electrophoresis was performed and silverstained genotypes bands were compared with standards (allelic ladder) and identified.

In that case, two STR systems (short tandem repeat) were used: HUMTHO1 and HUMVWF31/A. The Figure 3 shows the band pattern obtained from standard mixtures of alleles and A and B samples for HUMTHO1. The allelic ladder for this genotype test is composed of 7 bands (corresponding to 5, 6, 7, 8, 9, 9.3, 10 alleles). In a population, each individual has in his genome a combination of two of these 7 alleles=genotypes (included the combinations of twice the same allele). The frequencies of each genotype is known and published. (10, 11).

In Figure 3, A and B samples are exhibited an identical pattern of two bands, which corresponds to the allelic form 6 - 9.3.

The other allele system tested (HUMVWF31/A) was also showing an identical band pattern (alleles 14 and 17) for both samples.

In a caucasian pouplation, these alleles are present in the human genome at the following frequencies:

HUMTHO1 6-9.3	Genotype frequency:	15.0 %
HUMVWF31/A 14-17	Genotype frequency:	4.7 %

The allele combination frequencies in a caucasian population can thus be calculated:

$$15.0 \% \times 4.7 \% = 0.7\%$$

From these DNA-PCR analyses results, conclusions can be drawn as following:

1. There is no exclusion between A and B samples (no difference in allele bands in the two systems tested).
2. A genotype frequency of 0.7 % in a caucasian population means that 7 individuals over 1000 people will show the same genomic pattern.

Consequently, we cannot completely exclude the possibility that both urines have been produced by two different individuals, but this can be statistically calculated.

Other systems can of course be tested in order to lower the statistical frequency of the same genomic characteristics, then to increase the identification probability. This has not been successful in this case.

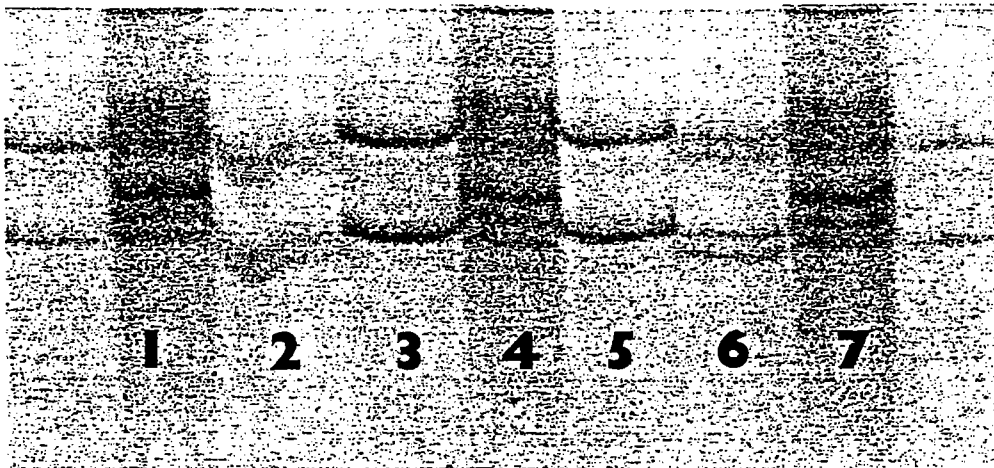


Figure 3: Silver stained HUMTH01 genotypes obtained after PCR and hydrolink gel electrophoresis.

1. Allelic ladder (5, 6, 7, 8, 9, 9.3, 10)
2. A-sample urine + genotype 5 - 9 *
3. A-sample
4. Allelic ladder
5. B-sample
6. B-sample + genotype 5 - 9 *
- 7 Allelic ladder

* Known genotype 5 -9 have been added in order to better identify the genotype 6-9.3

Urine storage conditions

Highly degraded urines are not suitable for DNA typing with PCR. In order to minimize the contamination by bacteria or fungi, the storage at 4°C is not sufficient. The Figure 4 shows that sodium azide could prevent the contamination, and consequently preserve the rate of success in typing the samples.

The best method of storage is certainly to freeze the pellet after centrifugation of the urine. The sodium azide does not seem to be essential in that case (see 8).

In the doping analyses conditions, sample centrifugation and freezing of the pellet right after the arrival of urine in the lab is feasible for A sample. Since the B-sample could not be open, freezing the entire B-sample would be the best solution in order to keep good chances to perform DNA typing. In the future and in any case, to identify the producer of a sample, a comparison with a fresh urine or with blood would be the best solution.

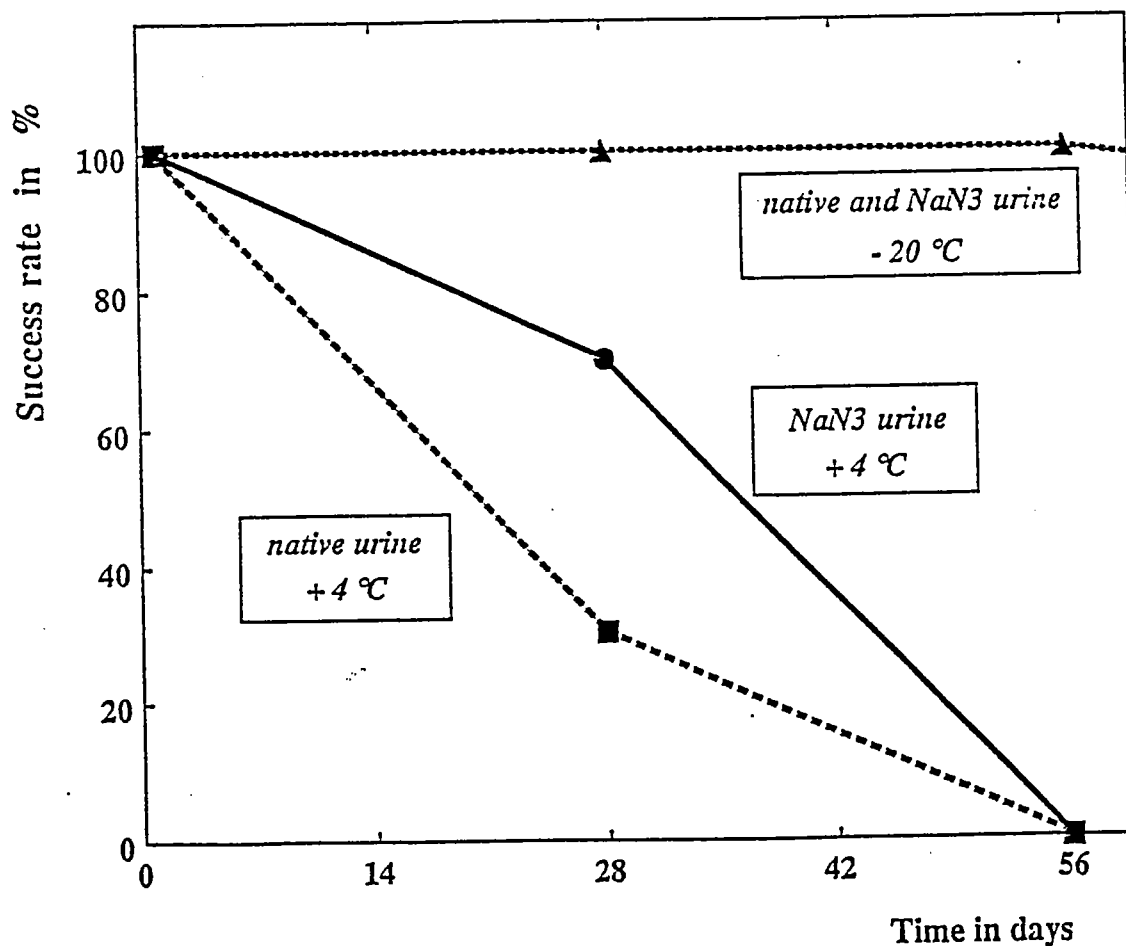


Figure 4: Amplification success rates for urines at different conditions of storage.(n=10)

Conclusions

1. PCR is a powerful tool for the genetic comparison between two or several urines.
2. The investigations are generally easier for female urine than for male urine.
3. Some precautions should be taken for the urine storage in order to get PCR success (bacteria contamination!)
4. The best way to identify the producer of the samples would be to get blood or fresh urine from the athlete.

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