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Validity of urine samples : microbial degradation

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Since several years, most often during summertime, we have observed the complete degradation of some urine samples upon arrival at the laboratory. Whatever conservation methods are used in the laboratory, the damages are irreversible and some analytical results are invalid.

The collection of the urine samples is not done under sterile conditions and it is possible that micro-organisms be present and multiply during transportation (delays) under favourable conditions and improper storage. Preliminary studies have permitted in some of these specimens, the identification of strains of *Enterobacteria* and *Staphylococcus*, but not in all, the micro-organisms being already dead. These micro-organisms are found in the gut microflora or on the skin, their presence in urine samples collected in these conditions is no surprise. However, several specimens contaminated by such microbes were not showing signs of degradation. On the other hand, contamination may occur in the laboratory namely from the water used. In the past years, some experts have raised the possibility of microbial formation of testosterone in positive athlete's urine sample to contest the validity of the laboratory findings¹. Their claim is that enzymes such as the 3(17)-hydroxysteroid dehydrogenases (HSDH) and the Δ^4 -5 β -dehydrogenase for example, are able to convert several steroids to testosterone and that such enzymes are widely found in the micro-organisms that can contaminate urine samples. Some potential sources of microbial testosterone were described in the sixties. For example, the conversion of 5 β -steroid to Δ^4 -steroid by the steroid Δ^4 -5 β -dehydrogenase was reported² and 3 β -HSDH and Δ^4 nuclear steroid dehydrogenase may convert saturated steroid to testosterone *via* 4-androsten-3,17-dione and 1,4-androstadiendione³.

¹ 1994-1995 Cases : D. Modahl, UK, A. Okeke, Norway, M. Gravelle, USA ; R.S. Bilton, *Lancet(letter)*, 345 (1995) 1186

² S.J. Davidson and P. Talalay et al., *JBC*, 241 (1966) p.906; *Enzyme Handbook*, T.E. Barman, Donnelley & Sons, Virginia, USA, (1969) p. 166.

³ R. S. Bilton *Lancet*, 345 (1995) 1186, *Enzyme Handbook*, T.E. Barman ed. (1969), P. Talalay et al. *JBC*, 218 (1956) 675.

We have shown from preliminary work and after detailed analysis of the urinary steroids, that when some contaminated samples were subjected to temperatures of 37°C during days, the steroid conjugates were hydrolysed and testosterone and other urinary steroids accumulated in the free fraction⁴(figure 1). Although we have not observed the alteration of the structure of the steroids (figure 2), the analysis of testosterone in the conjugated fraction was invalid. The increase of free testosterone in urine samples was also reported by Kjeld et al.⁵. The stability of steroid conjugates such as testosterone glucuronide in urine matrix was not studied extensively but it was shown and it is widely accepted, that their measurement is not impaired when the samples are stored at -20°C for up to one year⁶. We have observed that if the urine samples are not contaminated, the steroid glucuronides are stable under these conditions, while the more labile steroid sulfates such as epiandrosterone, androstendiol, dehydroepiandrosterone are hydrolysed to a certain extent.

In the presence of other micro-organisms, the effects were more important and cumulative(figure 3). In the cases observed, the major urinary steroids such as androsterone, etiocholanolone, epiandrosterone, testosterone and the androstandiols were extensively degraded and other steroids were formed and accumulated in the free fraction. These steroids were identified as the 5 α - and 5 β -androstan-3,17-dione (figure 4) usually present in trace amount in urine samples. After a several days, the natural steroids are undetectable (figure 5). The formation of 5 α -androstandione which may hide the epitestosterone combined with the degradation of testosterone and epitestosterone caused an apparent reduction of the T/E value in some chromatographic systems⁷(figure 6), DHT is an intermediate in the microbial conversion of androstandiol to androstandione. Under such conditions, the analysis of natural steroids such as testosterone, epitestosterone and DHT is invalid. Since early 1991, our screening procedure includes the detection of the androstandiones and urine specimens showing their presence are given as invalid.

⁴ C. Ayotte, A. Charlebois and D. Goudreault, *J. Chromatogr., Biomed. Appl.*, accepted for publication in January 1995

⁵ J.M. Kjeld, C.M. Puah and G.F. Joplin, *Clin. Chim. Acta*, 80 (1977) 285

⁶ K. Robards and P. Towers, *Biomed. Chromatogr.*, 4 (1990) 1; E. Venturelli, A. Cavalleri and G. Secreto, *Chromatogr., Biomed. Appl.*, 671 (1995) 363

⁷ CAP revision report (1992)⁷

Similar effects can be observed during the enzymatic hydrolysis. Some crude mixtures of *H. pomatia* juices are known to possess some unwanted enzymatic activities causing the degradation of the urinary steroids⁸. When confirming an elevated T/E value, the free fraction is now also analysed in order to verify the absence of testosterone in the free form. Although it was previously reported⁹, we never observed the formation of free testosterone in urine samples ; however, more systematic investigations will be done. Furthermore, the measure of an elevated urinary pH value, although indicating the ageing of the sample, cannot in itself invalidate the analysis of a sample. Only half of the specimens of very high pH values (between 8 and 9) have shown signs of degradation invalidating the measure of the T/E value (figure 7) . Conversely, some heavily degraded samples were showing normal pH values.

The formation of the $5\alpha/\beta$ -androstandiones or the deconjugation of the urinary steroids are not observed when samples are stored at -20°C to 4°C even for prolonged periods of time. Both reactions are inhibited by bacteriostatic agents such as sodium azide and mercuric chloride added in concentrations of 0.5%. The agents must be added few hours before the incubation to effectively precipitate proteins. Lowering the pH is not necessary and may cause the hydrolysis of the steroids sulfates such as HgCl_2 does.

Conclusion

We are now investigating more completely the bacterial contamination of urine samples. We will make a repertoire of the microbes found in specimens and of their effects. Inoculations of sterile urine samples and of culture media containing authentic steroids standards should provide more definitive answer, especially concerning the real possibility of forming testosterone in the samples. However, at this moment, urine samples collected under the present conditions are very often contaminated by microbes but this will not necessarily lead to the degradation of the specimen. Both degradation processes are stopped when samples are stored at temperatures below -4°C . Improvement of the transportation to the laboratory can solve the problem since even at 37°C , nearly four days are needed to observe the signs of degradation. Instructions must also be given to

⁸ M. Donike et al., R. Massé, C. Ayotte and R. Dugal, *J. Chromatogr.*, 489 (1989) 23 ; E. Vanluchene, W. Eechaute and D. Vandekerckhove, *J. Steroid Biochem.*, 16 (1982) 701

⁹ H. Geyer et al., Proceedings of the 13th Workshop on Dope Analysis, (1996) p.95.

doping control officers to insure a proper refrigeration of the specimens and this may prevent the addition of a bacteriostatic agent. The laboratory should report the results as invalid when signs of degradation are present.

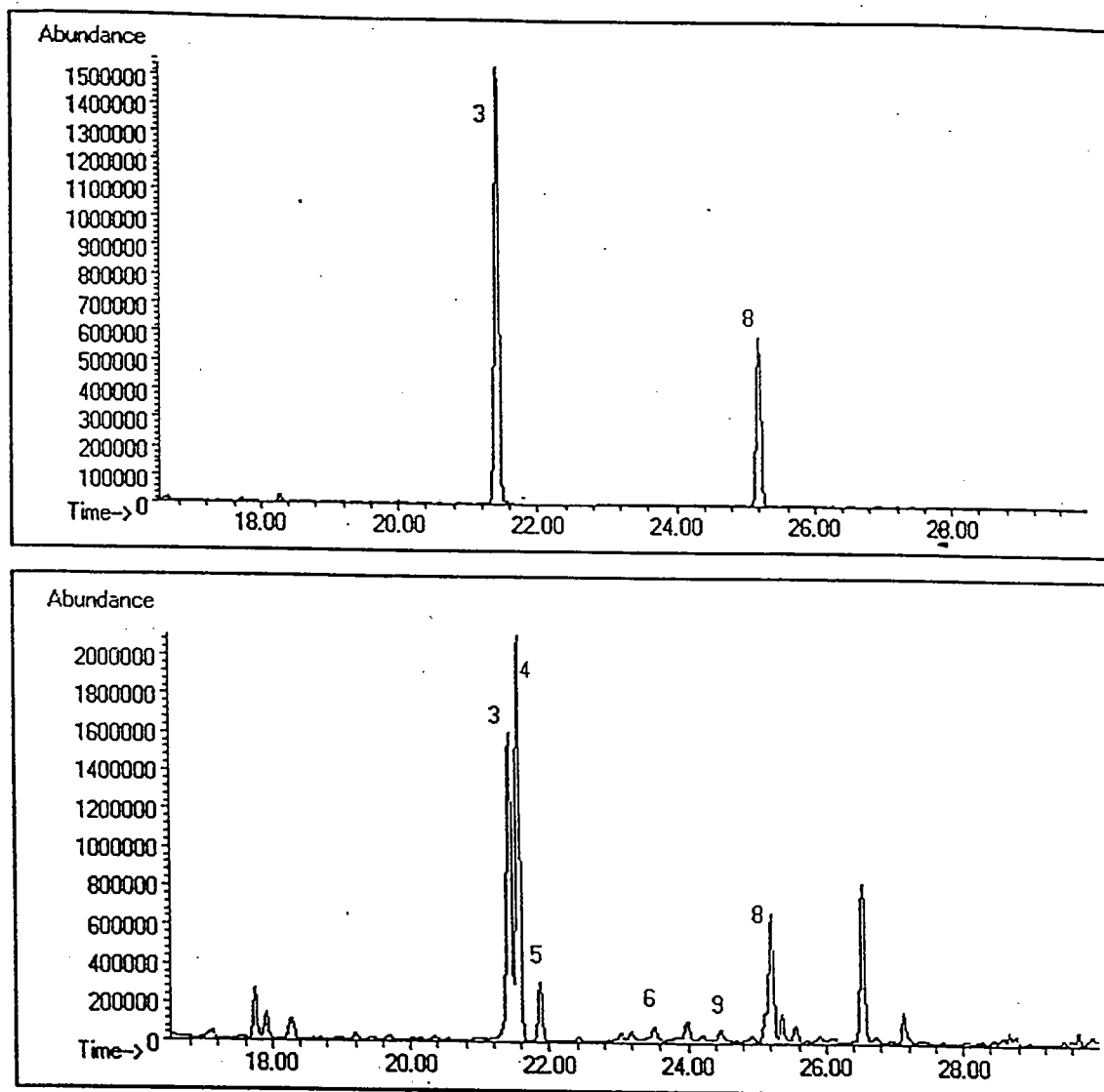


Fig. 1 - Free fraction of a urine sample contaminated by bacteria (not identified) and incubated at 37°C for 24 hours (upper panel) and 120 hours (lower panel). Peak 3 : epietiocholanolone (ISTD), 4 : androsterone and etiocholanolone, 5 : 5 α , β -androstan-3 α , 17 β -diol, 6 : epitestosterone, 8 : ISTD, 9 : testosterone

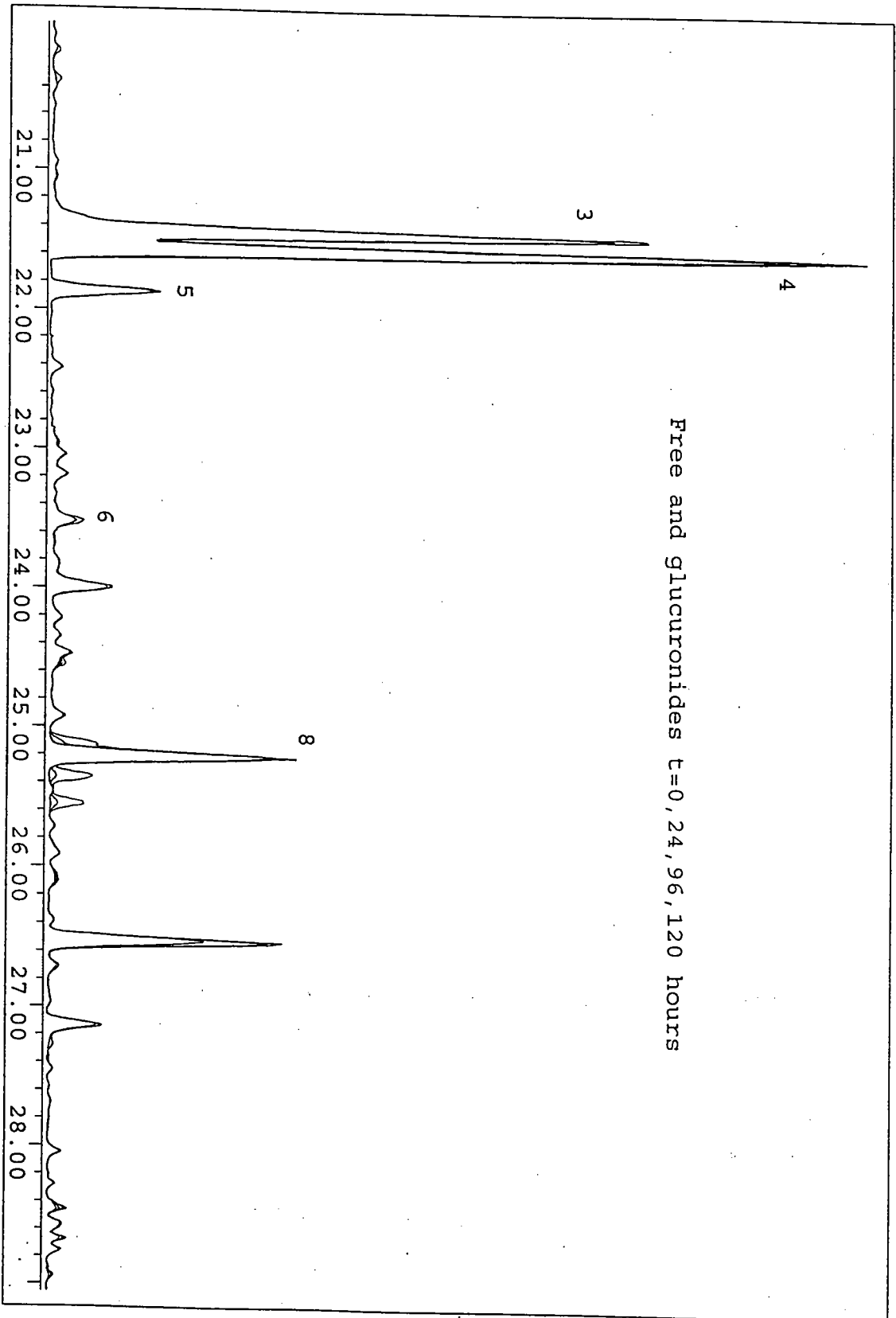


Fig. 2 - Urine sample contaminated by bacteria (not identified) and incubated at 37°C.

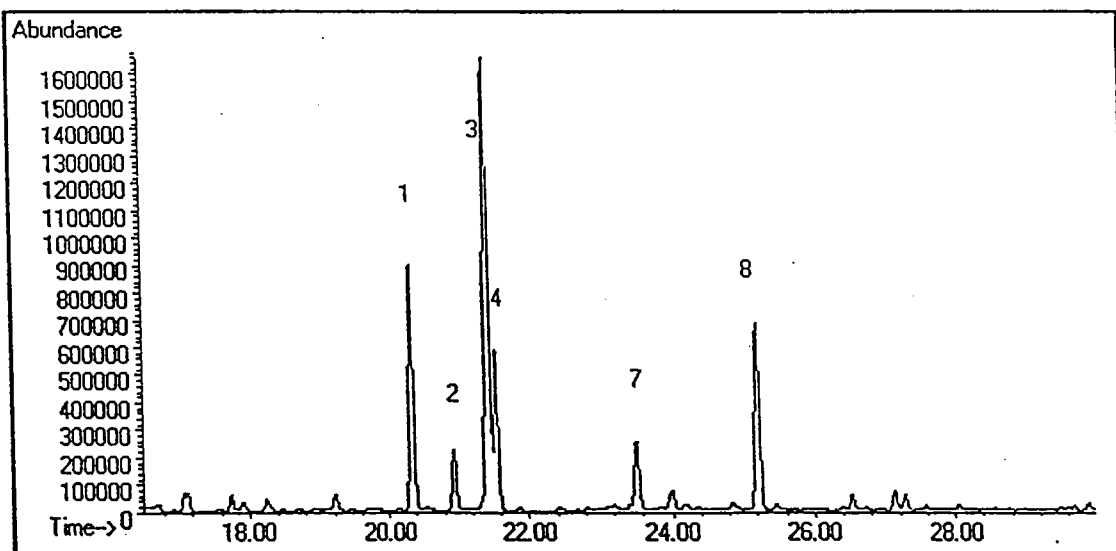
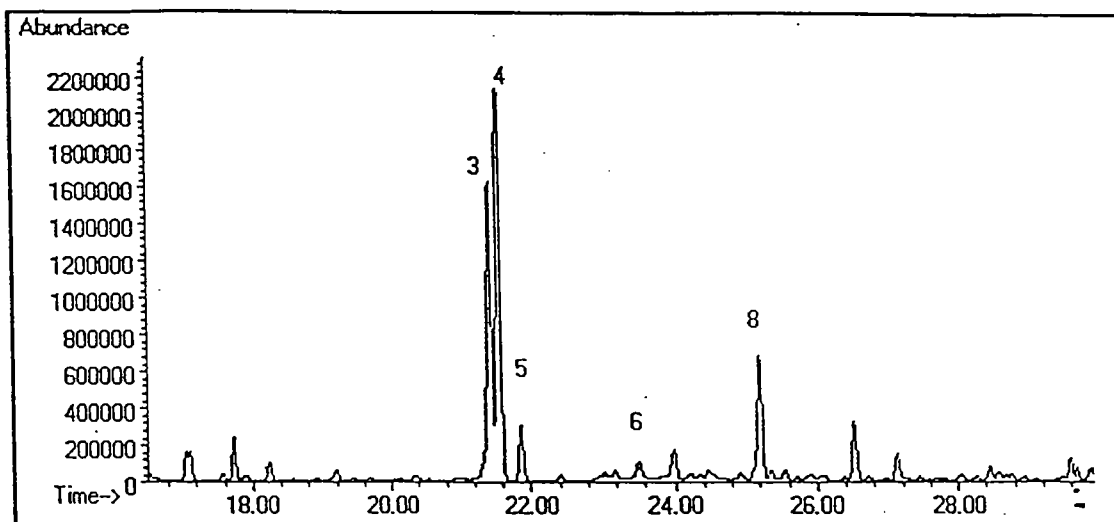


Fig. 3 - Urine sample inoculated with bacteria (not identified) (free and glucuronide fractions) and incubated at 37°C for 6 hours (upper panel) and 120 hours (lower panel). Peaks 1, 2 : 5 β -androstan-3,17-dione, 3 : epietiocholanolone (ISTD), 4 : A and Etio, 5 : 5 α , β -androstan-3 α ,17 β -diols, 6 : epitestosterone, 7 : 5 α -androstan-3,17-dione, 8 : ISTD

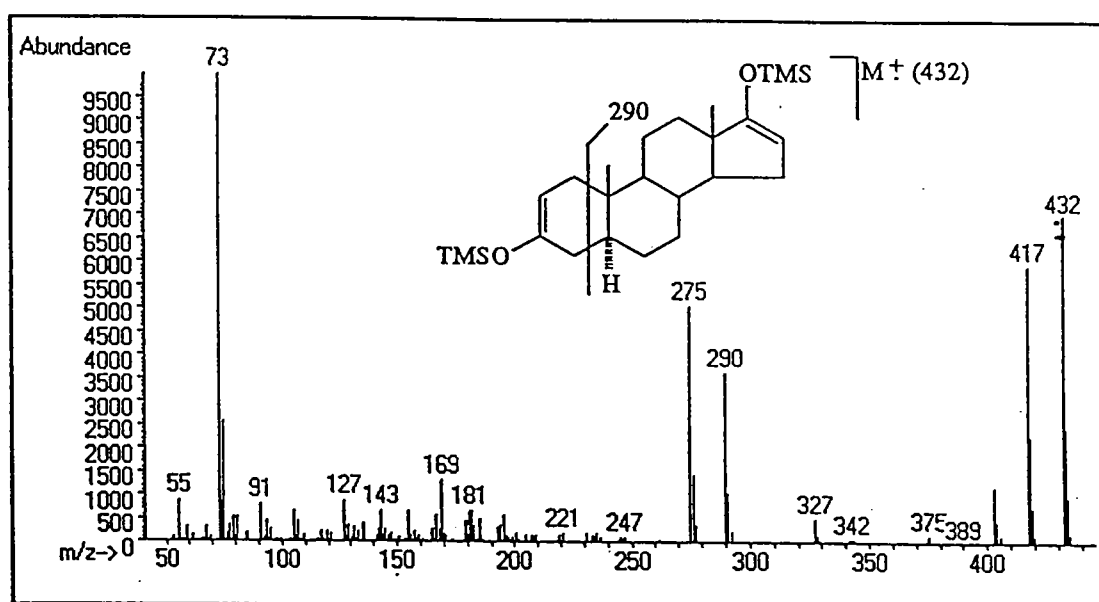


Fig. 4 - Mass spectrum of the TMS-enol derivative of 5 α -androstan-3,17-dione obtained from the GC/MS analysis of the authentic standard (20 ng injected).

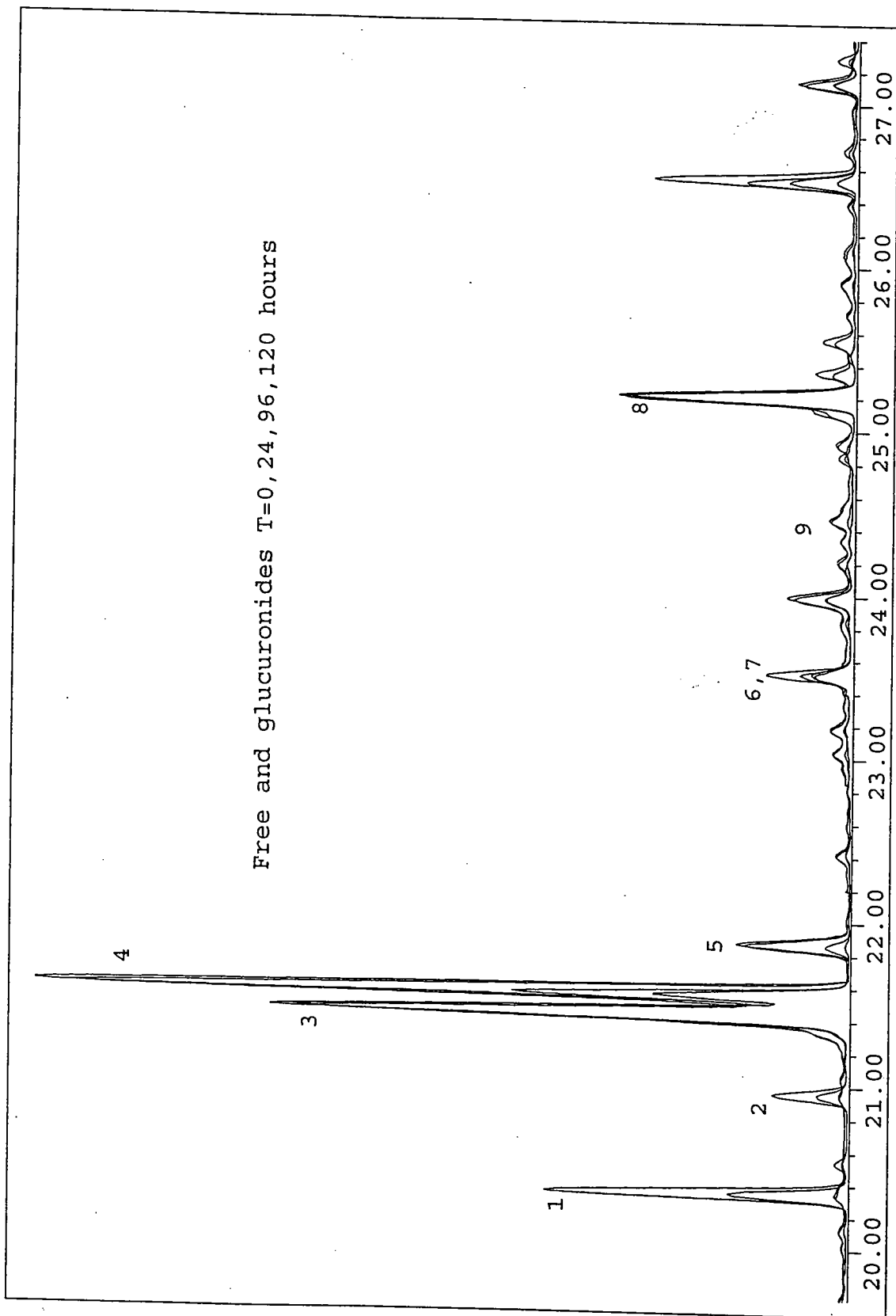


Fig. 5 - Urine sample (50 mL) inoculated with 1.0 mL of a sample containing bacteria. Incubations done at 37°C.

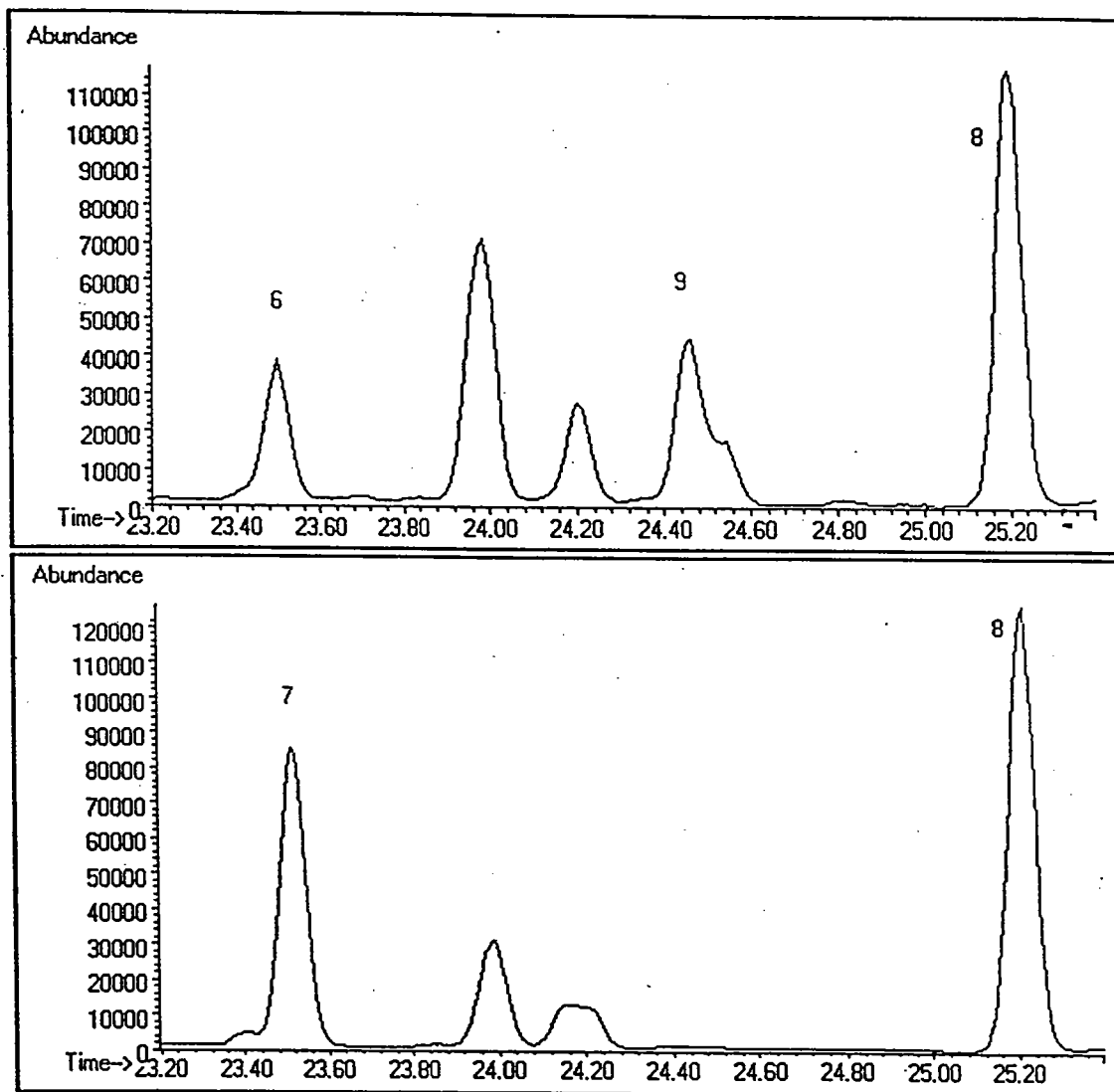


Fig. 6 - GC/MS analysis (432.4 and 435.4 ion chromatograms) of the free and glucuroconjugated fractions of a urine sample contaminated by bacteria, 6 hours after inoculation (upper panel) and 120 hours after (lower panel). Peaks 6 : epitestosterone, 7 : 5α -androstan-3,17-dione, 8 : 17α -methyl- 5α -androstan-3 β ,17 β -diol (internal standard), 9 : testosterone.

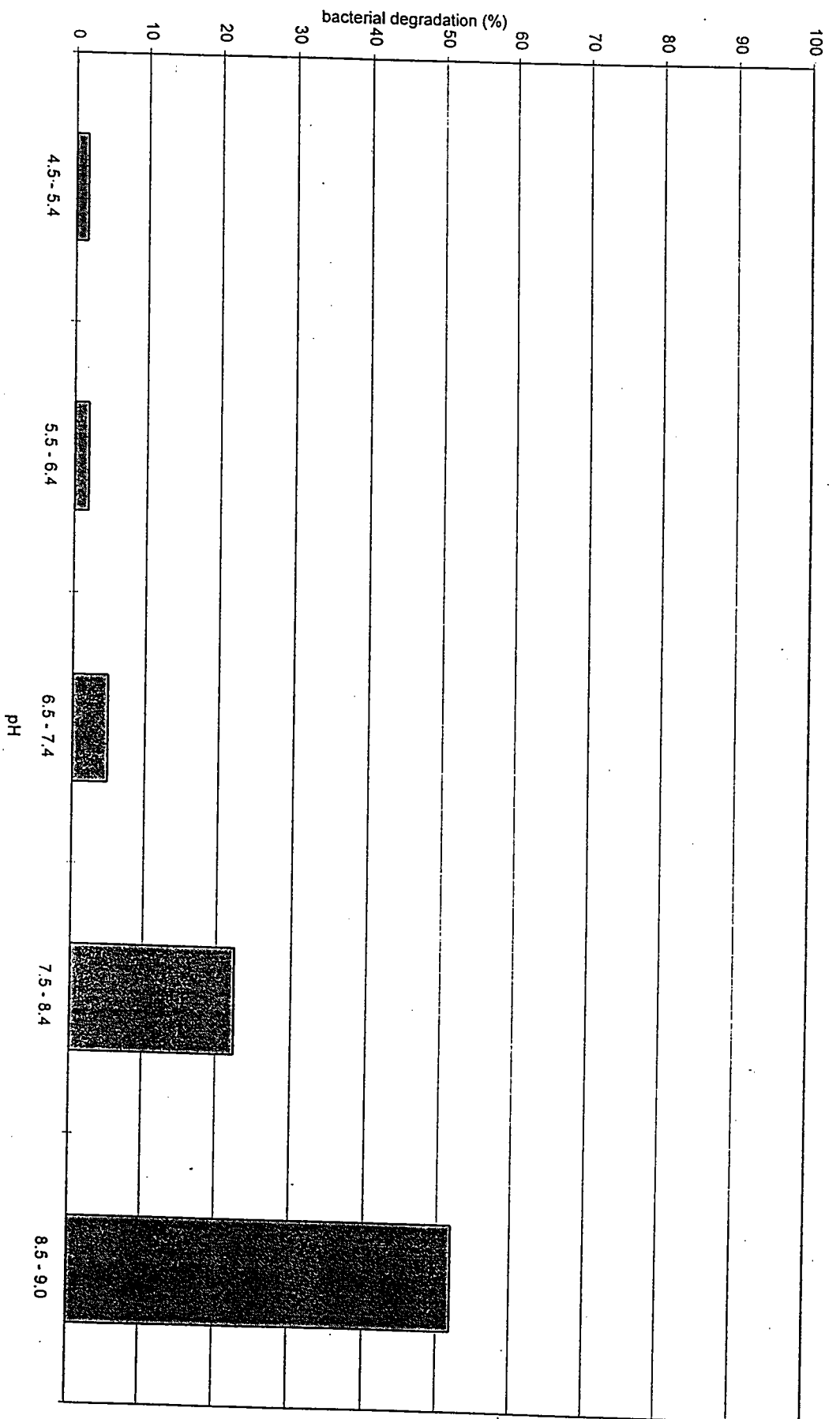


Fig. 7 - Percentage of samples showing signs of bacterial degradation in relation with the pH values of specimens received from doping control programs (1994-1995).