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Bacterial proteases in urine samples – effects on EPO analysis?

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Introduction

Proteolytic degradation may affect or even impede the detection of peptide hormones in urine samples, e.g. EPO. The intentional manipulation of control samples by addition of proteases during or after sampling was confirmed in several studies. To deal with this problem, subsequent investigations suitable to detect proteolytic activity or to identify the present enzymes in urine are necessary.

Beside the exogenous origin, other sources of proteases in urine samples have to be taken into consideration. Thevis et al. investigated the urinary excretion of endogenous proteases in 120 routine doping control samples [1]. 95 % of these samples contained a protease activity of less than 6 µg/mL (concentrations equivalent to trypsin), representing a significant lower quantity compared to the expected amount in adulterated urines (20 µg/mL in minimum). To differentiate suspicious samples, a threshold of 15 µg/mL was proposed to determine elevated protease activity after fraudulent urine manipulation [1].

Furthermore, several bacteria causing urinary tract infections (UTI) are known to synthesise proteases, e.g. *Escherichia coli, Proteus mirabilis,* and *Klebsiella pneumoniae* [2-4]. The present study aimed at the investigation of possible influences of microorganisms on the stability of EPO in urine samples and initially focused on the following questions:

- Do uropathogenic bacteria release proteases in urine samples, and in which amount?
- Would an UTI affect the analysis of EPO in urine for doping control?

To investigate the possibility of the production of relevant protease concentrations, urine samples spiked with rEPO and NESP, respectively, were incubated in the presence of selected bacterial species. Growth of bacteria was checked by semi-quantitative subculture. Following incubation, protease activity was measured using a protease fluorescent detection kit and EPO was investigated by IEF analyses.

Materials and Methods

Incubation experiments

The test urine (blank, female volunteer) was spiked with rEPO (23 pg/mL) and NESP (12 pg/mL), respectively, aliquoted and sterile filtered.

Test urines were inoculated with three different bacteria species (*E.coli, P.mirabilis, K.pneumoniae*, resp.; 104-105 CFU/mL each), and protease control samples were prepared by addition of trypsin (10 µg/mL). Incubation was carried out at 37°C for 24 hours. As negative controls, sterile urine aliquotes were incubated in the same manner. All samples were processed in duplicate.

After incubation, bacterial growth was checked by semi-quantitative subculture (Columbia blood agar by OXOID). Subsequently, samples were sterilized by filtration and kept frozen until further processing.

Protease activity

Protease activity in urine samples was investigated by a protease fluorescent detection kit (Sigma), utilizing a fluorescence detector (Shimadzu RF-551). Samples were analysed according to the assay instructions. To achieve an increased sensitivity, reaction time for the cleavage of the fluorescein isothiocyanate-labeled casein substrate was extended up to 3.5 hours. Protease concentrations were estimated in comparison to control solutions containing trypsin (100, 250 ng/mL and 10 µg/mL).

EPO analyses

The urine samples were processed by IEF analysis according to the standard EPO protocol (IEF-double blotting and chemiluminescent detection) [5].

Results and Discussion

Bacterial growth

Following the 24 hours-incubation, inoculated samples showed distinctive characteristics of UTI urines (turbidity, odour). Semi-quantitative determination of colony forming units (CFU) confirmed the bacterial growth during incubation (104-105 and >105 CFU/mL, resp.).

Protease activity

Samples incubated in the presence of *K.pneumoniae* gave no significant fluorescence reading, according to the specification of the assay. Urines inoculated with *E.coli* and *P.mirabilis*, respectively, resulted in significant signals of the fluorescent detection kit. However, the protease activities were rather low and concentrations were estimated between 100 and 250 ng/mL (compared to trypsin control solutions).

Lamon et al. investigated the influence of low protease concentrations and demonstrated the stability of EPO in urine against 0.5 µg/mL of trypsin (incubation overnight) [6]. Therefore, the quantity of detected bacterial proteases in the present study might be insufficient to cause interferences with IEF analysis.

IEF analyses

Considerable effects occurred in urine samples inoculated with *K.pneumoniae* and *E.coli*, respectively (see Figure: lanes 3/9; 5/11). Bacterial influences resulted in a shifting in the position of the EPO bands, smears or even erasure of signals.

According to the WADA Technical Document TD2007EPO [7], the identification criteria for an AAF as well as the acceptance criteria have not been fulfilled in samples containing *K.pneumoniae* and *E.coli*, which are therefore not evaluable regarding the EPO profile.

The presence of *P.mirabilis* did not affect the detection of rEPO and NESP by IEF analysis (see Figure: lanes 4/10).

Conclusions

Two common bacterial species causing UTI (*E.coli, K.pneumoniae*) have been shown to be able to influence IEF analysis of rEPO and NESP in urine and lead to undetectable EPO profiles.

Protease activities were only measured in samples containing *E.coli* and *P.mirabilis*. In contrast, IEF signals of rEPO and NESP were only affected in urines incubated in the presence of *K.pneumoniae* and *E.coli*. Thus, IEF results in urine samples were not in all cases consistent with the detection of protease activity; accordance was only determined for *E.coli*. The results suggest that in addition to the presumed proteolytic activity other structural changes are caused by UTI bacteria, e.g. alterations at the glycan chains of the EPO molecule, taking also into account the relatively low protease concentrations. Further studies are in progress to estimate the issue of uropathogenic bacteria and the resulting consequences for doping control.

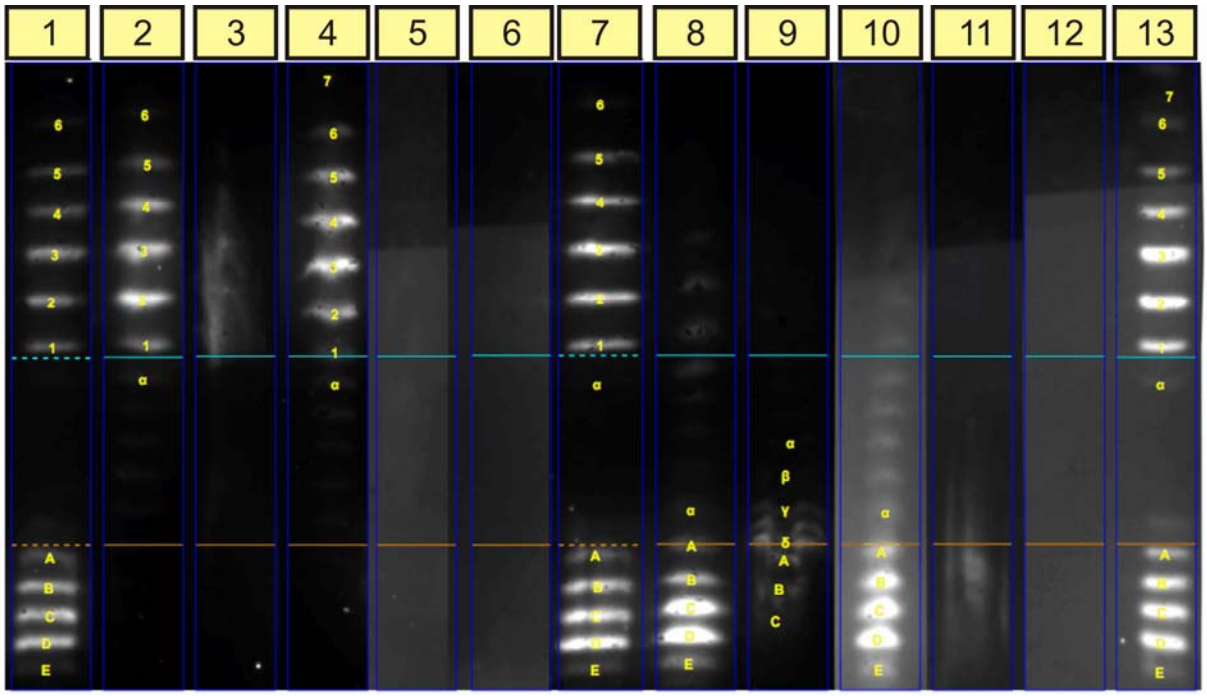

Figure: IEF analyses of test urines containing rEPO and NESP, resp., following the incubation in presence of selected UTI bacteria (24 hours, 37°C).
Alterations of EPO were observed for *K.pneumoniae* (lanes 3/9; in contrast to the absence of a protease activity determined by the fluorescence detection kit), and *E.coli* (lanes 5/11; consistent with the presence of a protease activity). Incubation of urines with *P.mirabilis* did not influence IEF analysis (lanes 4/10), although protease activity was measured.
Lanes 3-5: test urines spiked with rEPO and inoculated with *K.pneumoniae, P.mirabilis, E.coli*, resp.; lanes 9-11: test urines spiked with NESP and inoculated with *K.pneumoniae, P.mirabilis, E.coli*, resp.; lanes 6, 12: "protease positive" test urines, incubated after addition of trypsin (10 µg/mL); lanes 2, 8: sterile test urines spiked with rEPO and NESP, resp., incubated without addition of bacteria; lanes 1, 7, 13: quality control samples (rEPO, NESP).

References

[1] Thevis M, Maurer J, Kohler M, Geyer H, Schänzer W. (2007) Proteases in doping control analysis. *Int J Sports Med* **28**, 545-9.
[2] Maurizi MR. (1992) Proteases and protein degradation in Escherichia coli. *Experientia* **48**, 178-201.
[3] Wassif C, Cheek D, Belas R. (1995) Molecular analysis of a metalloprotease from Proteus mirabilis. *J Bacteriol* **177**, 5790-8.
[4] Trishin AV, Zhdanovich MIu, Savvateeva LV, Toptygin AIu, Donenko FV, Kiselevskiĭ MV, Kurbatova EA, Gruber IM, Elkina SI, Kalina NG. (2004) Protease activity of Klebsiella pneumoniae of different virulence. *Zh Mikrobiol Epidemiol Immunobiol* **4**, 7-11.
[5] Lasne F. (2001) Double-blotting: a solution to the problem of non-specific binding of secondary antibodies in immunoblotting procedures. *J Immunol Methods* **253**, 125-31.
[6] Lamon S, Robinson N, Sottas PE, Henry H, Kamber M, Mangin P, Saugy M. (2007) Possible origins of undetectable EPO in urine samples. *Clin Chim Acta* **385**, 61-6.
[7] World Anti-Doping Agency. (2007) WADA Technical Document TD2007EPO (Version 2.0).