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Development of a high temperature LC method for hormone analysis

Eva de Rijke, Paul Zoontjes, Hennie van Rossum, Saskia Sterk

Introduction

With conventional LC-MS, endogenous hormones such as testosterone (T) and estradiol (E) cannot be discriminated from their illegally administered (natural) analogues in urine. This is, however, possible with isotope ratio mass spectrometry (IRMS), which determines the difference between the carbon isotope ratio ($^{13}C/^{12}C$) of the endogenous and exogenous hormones. In this study, a high temperature liquid chromatography (HTLC) method for T and E and their precursors was developed for future coupling with IRMS. Because of low UV absorption for some hormones, a QToF-MS system was included in the set-up via a 1:15 splitter and MeOH was added before the MS to enhance ionization, as shown in Figure 1. In LC-IRMS no organic solvents (source of carbon) can be used because they influence the isotope ratio of the analytes. Therefore, a high temperature gradient separation with 100% water as an eluent was developed.

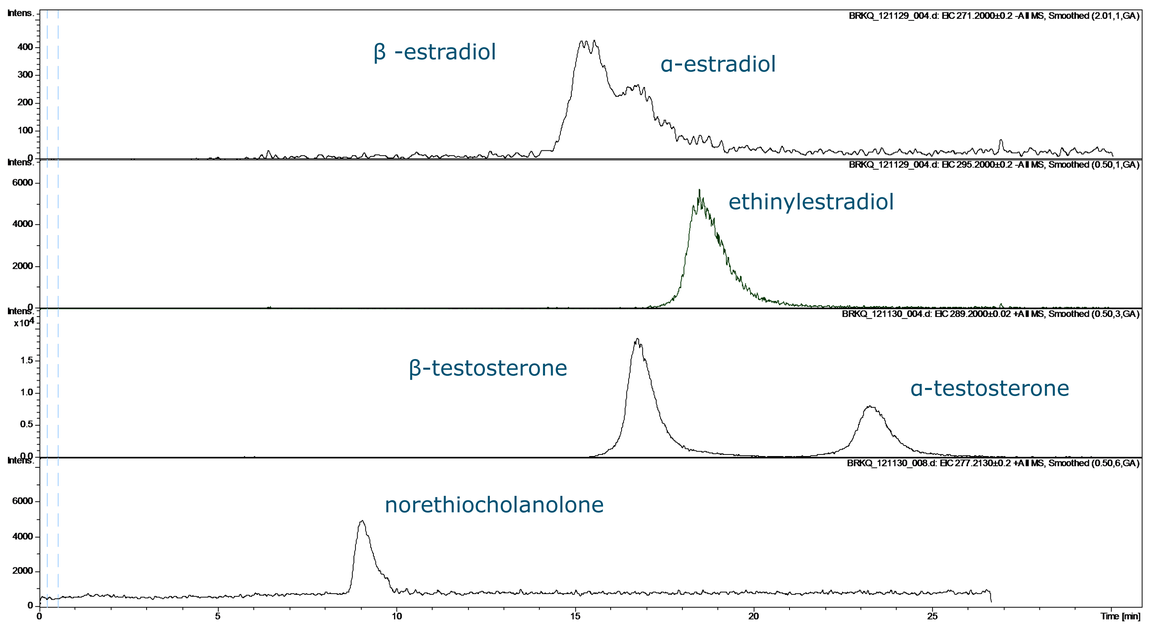

Figure 3. HTLC separation of hormones in bovine urine (Zirchrom 100x2.1 column, 0.3 ml/min).

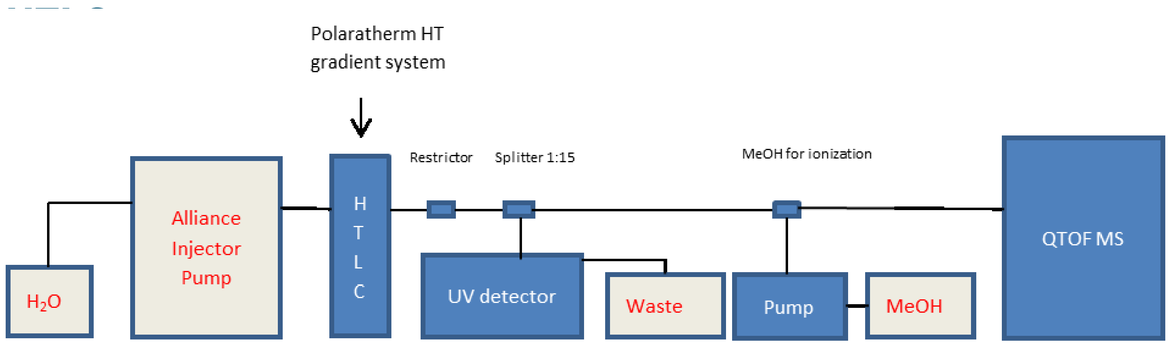

Figure 1. Schematic overview of HTLC-UV-QToF-MS set-up.

Column selection and HTLC gradient optimization

Four columns were tested using α- and β-T standard solutions: a Hamilton PRP-1 (150x3.0), a Supelco TRP-100 (200x3.0), an Agilent Extend C_{18} (100x2.1) and two Zirchrom ZR-PBD columns (100x4.6 and 100x2.1). The Zirchrom columns performed best on stability and resolving power at high temperature. The 4.6 mm I.D. column performed best at a flow of 3 ml/min (Fig. 2A), but this is too high for IRMS coupling. The 2.1 mm I.D. column could be operated at a lower flowrate of 0.3 ml/min, which is compatible with IRMS. The best results were obtained with a 10 ºC/min gradient, from 60 ºC to 120 ºC with a 10 min hold time (Fig. 2B).

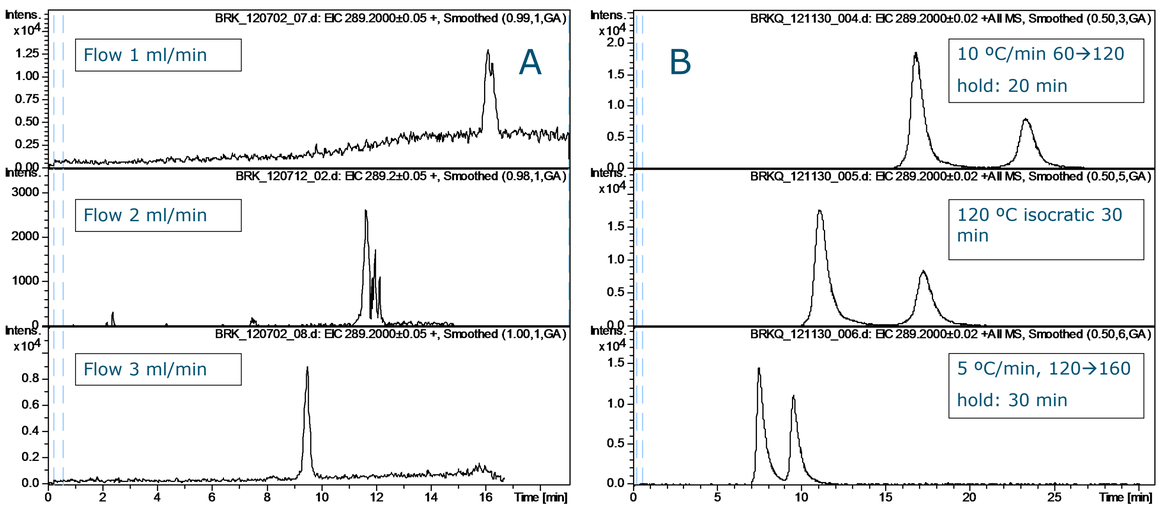

Figure 2. HTLC gradient optimization with α/β-T on two Zirchrom columns (A) 100x4.6 mm, HT gradient: 60→120ºC, 10 ºC/min, hold 10 min (B) 100x2.1 mm, flow: 0.3 ml/min.

Application: T and E in human and bovine urine

Two bovine urines, a humane urine and a control water sample, fortified with 10 ng/µL α/β-T, α/β-E, norethiocholanolone, ethinylestradiol, 5-androstene-3ß,17α-diol, 5α-androstane-3ß,17α-diol, were analyzed using the optimized conditions. The samples were purified on a StrataX C_{18} SPE column and injected on the HTLC system. The results are presented in Figure 3: α/β-T and norethiocholanolone show a good separation and MS response in +ESI, while α/β-E and ethinylestradiol show a better response in –ESI. 5-androstene-3ß,17α-diol and 5α-androstane-3ß,17α-diol are not detected with HTLC-MS, as they do not ionize very well under non-acidic conditions. To determine their recovery, one fraction (8-25 min) containing all compounds from the standard was collected and consequently derivatized with TMS and quantified with GC-MS/MS. For all compounds (including 5-androstene-3ß,17α-diol and 5α-androstane-3ß,17α-diol) recoveries were close to 100%, except α-T which had a much lower recovery.

Conclusions and discussion

- The first results of this high temperature LC study with 100% water as a solvent are promising
- All hormones of interest can be resolved
- The Zirchrom ZR-PDB column performed best on stability and high temperature resolving power
- Relatively long gradients and broad peaks compared to conventional (UP)LC are not a problem as the method is not designed for screening, but for comfirmation analyses
- HTLC can be used for future LC-IRMS analyses of a selected set of hormones

Acknowledgement

This project was financially supported by the Dutch Ministry of Economic Affairs, Agriculture and Innovation (project 72708.01).

RIKILT Wageningen UR
P.O. Box 230, 6700 AB Wageningen, The Netherlands
Contact: eva.derijke@wur.nl
T + 31 (0)317 48 03 52, F +31 (0)317 41 77 17
www.wageningenUR.nl/en/rikilt