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Research article

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Development of a second-generation Testosterone synthesis route via biocatalysis

Dirk-Jan van Zoelen[1*], Mellany van Heijningen-Ramaekers[1], Wouter Hoeberichts[2], Luuk Maartense[2], Jan Ytzen van der Meer[2], Stock, H.T[1]

[1]*Aspen Oss B.V. Kloosterstraat 6, 5349AB Oss, The Netherlands*

[2]*Currently employed elsewhere*

[*]Corresponding Author
Dirk-jan van Zoelen, Aspen Oss B.V. Kloosterstraat 6, 5349AB Oss, The Netherlands.

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Abstract

Testosterone is a male hormone which is being manufactured in pharmaceutical industry for many years. Testosterone is the primary sex hormone and anabolic steroid in males. It is also used as a drug to treat male hypogonadism, gender dysphoria, bone loss, certain types of breast cancer, prostate cancer and hypersexuality [01]. It may also be used to increase athletic ability in the form of doping. Most of the time the current manufacturing routes start from 4 androstene 3,17 dione which is chemically converted to Testosterone by a reduction reaction. In this article we present the development of a second-generation route towards Testosterone via Biocatalysis, using an oxidoreductase enzyme. This results in a more sustainable API Testosterone. The overall PMI decreases from 69 to 44. Consequently, the enzymatic route reduces the environmental impact based on material use by 36%. Via proteomics principles we have been able to develop a generally applicable in-house analysis/method to prove absence of residual enzyme with a detection limit as low as 1 ppm.

Keywords: Testosterone, Biocatalysis, Sustainability, Proteomics

Introduction

For millions of years nature has evolved, and many complicated chemical conversions are carried out by enzymes, resulting in very efficient processes. For example, the human body uses enzymes to digest food and to build hormones such as progesterone, estradiol or Testosterone [01]. Moreover, plants have also become very efficient in producing all kinds of alkaloids via enzymatic processes.

In the design of a route towards an Active Pharmaceutical Ingredient (API), by means of traditional organic chemistry, additional process steps are often needed (e.g. application of protecting groups followed by a deprotection step). Typically, the use of purification steps is also required because via traditional synthetic methods, stereocenters are usually not introduced selectively. Various impurities can arise depending on the applied chemistry. Furthermore, traditional synthetic organic methods sometimes require the use of reactive or toxic chemicals and elevated temperatures. The use of enzymes in a synthesis route towards an API can lead to a more cost efficient, safer and more sustainable process.

Materials and methods

Enzymatically produced Testosterone was dissolved in a digestion buffer containing 1 ppm of labelled enzyme digestion fragments. The mixture was denatured, the digestion enzyme solution was added, and the mixture was incubated at 37°C overnight to digest residual enzyme. The digestion was quenched with TFA and the mixture was analysed by UHPLC-MS on presence of digestion fragments. The fragments were quantified against the labelled reference standards.

A Thermo Vanquish Flex UHPLC system was used in combination with a Thermo Q-Exactive Orbitrap High resolution mass spectrometer.

Enzyme used for digestion is obtained from Fisher Scientific. Enzyme used for conversion of 4-AD was obtained from Cambrex. Co-enzyme was obtained from Syncozymes. Peptide fragments for the protein analysis were purchased from Pepscan.

Results

At present, Testosterone is produced by Aspen API via a synthetic route comprising of two conversions and a purification step, starting from 4 androstene 3,17 dione via the intermediate androstenedione ethyl-enolether (cf. figure 1). A new route to Testosterone

has been developed at Aspen Oss, in which the same, globally accepted Regulatory Starting Material (RSM) 4 androstene 3,17 dione is enzymatically converted to crude Testosterone in a single step using an oxidoreductase enzyme and NADP as co-enzyme. For removal of residual biomaterial originating from the applied enzyme, a small silica plug was applied in the final purification. The product was purified by crystallization from acetone/water.

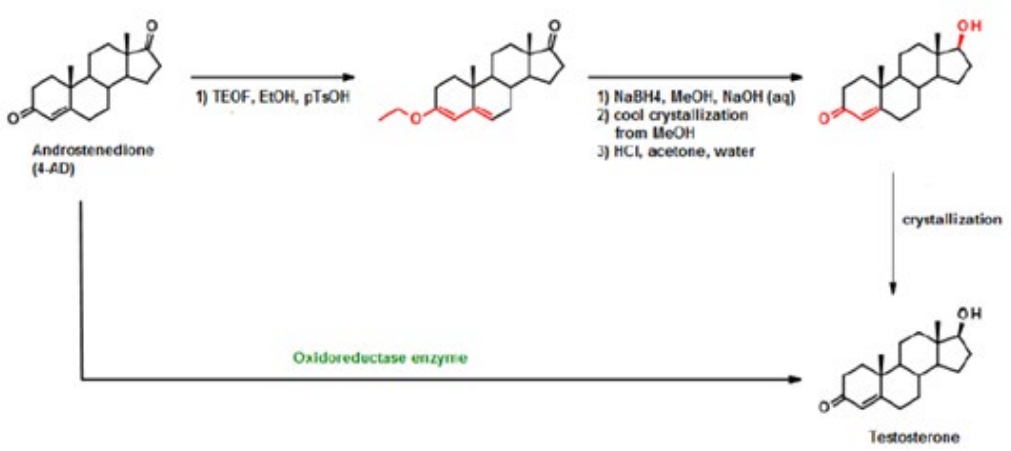

Figure 1: Classical chemical synthesis towards Testosterone and the second-generation route via the enzymatic pathway.

To define the environmental impact of a synthetic route, a Life Cycle Assessment (LCA) can be performed. One of the most useful green metrics is the Process Mass Intensity (PMI) ; this metric considers the amount of material needed to produce 1 kg of product. The PMI calculation for the current route to Testosterone resulted in an overall PMI of 69, which means that 69 kg of material is needed to produce 1 kg of Testosterone API. The PMI calculation for the enzymatic route to Testosterone resulted in a PMI of 44, which means that 44 kg of material is needed to produce 1 kg of Testosterone API. Consequently, the enzymatic route reduces the environmental impact based on material use with 36%. The water PMI (enzymatic route: 10, chemical route: 34) represents the highest contribution. Since water waste in API processes is difficult to recover and very energy consuming to destroy, the reduction in water consumption is another environment friendly improvement in the enzymatic route to Testosterone.

Since enzymes are used, endotoxins, residual DNA but also residual enzyme need to be analyzed. Endotoxins can be analyzed by standard endotoxin tests, whereas residual DNA can be analyzed via PCR testing. Proving the absence of residual enzyme proved to be a very challenging task. Via proteomics principles (digestion of the enzyme into known fragments and analysis by means of HPLC-MS of the fragments and quantification by means of internal standards) we have been able to develop a generally applicable in-house analysis/method to prove absence of residual enzyme as low as 1 ppm. Regarding the quality of Testosterone API a total purity of >99,5% was achieved with all specified impurities ≤ 0.10 m/m%, Δ6-TT: ≤ 0.20 m/m%, Any other impurities ≤ 0.10 a/a%.

Discussion

The most important part of this work consists of the removal of the biomaterial and the prove of absence of biomaterial in the final API. The presence of biomaterial can be investigated by means of several methods [2-8]. Standard methods like LAL test (Endotoxins), PCR (DNA/RNA) can be used. The presence of residual proteins appeared to be more troublesome. Methods like ICP, Lowry, HPLC and CBQCA resulted in too high limits or interference with Testosterone. Via proteomic principles a new method was developed which could detect residual proteins as low as 1 ppm.

Conclusion

The process described above has been executed on large scale development batches within our multipurpose multiproduct plant and is currently being validated. By applying enzymes, we have successfully developed a new route towards Testosterone which is safer (no HCl and NaBH4 is used anymore), more sustainable (decrease of PMI) and more cost competitive. The application of enzymes is currently being investigated on multiple other APIs within the portfolio of Aspen API.

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