European Pharmacopoeia Approach to Testing for Pyrogenicity

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Introduction

The texts of the European Pharmacopoeia (Ph. Eur.) play a major role in ensuring the quality of medicines in Europe. They consist in general chapters and monographs, which are mandatory quality standards ubiquitously applied by the licencing authorities of the 39 signatory countries of the European Pharmacopoeia Convention and the European Union, with the overall aim of protecting public health. The European Pharmacopoeia Commission, the decision-making body of the Ph. Eur., is responsible for the elaboration and maintenance of its content. The European Directorate for the Quality of Medicines & HealthCare (EDQM) is a directorate of the Council of Europe and is entrusted with publishing the Ph. Eur. and bringing these standards to its users.

It goes without saying that any official standards dealing with the quality of medicines must address the issue of potential contaminants in the products concerned. Medicinal products contaminated with pyrogenic substances and administered parenterally may cause adverse reactions ranging from fever to life-threatening shock-like symptoms. The aim of pyrogenicity testing is to limit, to acceptable levels, the risk of these adverse reactions happening.

In the Ph. Eur., medicinal products are tested for pyrogenic substances according to general chapter *2.6.8. Pyrogens*. The test consists of measuring the rise in body temperature induced in rabbits by the intravenous injection of a sterile solution of the substance to be examined. The chapter was first published in the Ph. Eur. in 1971 and is still prescribed in a large number of monographs and general chapters.

Endotoxins from gram-negative bacteria (lipopolysaccharides) are the most common cause of pyrogenic reactions induced by contaminated pharmaceutical products. The level of

bacterial endotoxins is verified using the procedures described in Ph. Eur. general chapters *2.6.14. Bacterial endotoxins* or *2.6.32. Test for bacterial endotoxins using recombinant factor C*, published for the first time, respectively in 1987 and 2020. These are the analytical methods most commonly used to address the pyrogenicity of medicinal products administered parenterally. They present the great advantage of avoiding the use of laboratory animals but the drawback of not detecting fever-inducing substances other than bacterial endotoxins.

There are, indeed, a small number of pyrogens that possess a different structure and that cannot be detected using the test for bacterial endotoxins. Such pyrogenic substances are detected using the procedures described in the general chapter *Monocyte-activation test (2.6.30)*. The monocyte-activation test is therefore an *in vitro* pyrogen test that has the advantage not only of avoiding the use of laboratory animals, but also of being able to detect any pyrogenic substance, i.e. both endotoxin and non-endotoxin pyrogens.

Replacement of the Rabbit Pyrogen Test

The Council of Europe's *European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes* was opened for signature in 1986. Since that time, the Ph. Eur. Commission and its experts have carried out a program of work committed to Replacing, Reducing and Refining (3Rs) the use of animals for test purposes. Achievements have been significant,[1] but there are still challenges ahead. The Convention is referred to in a number of Ph. Eur. texts, including chapter *2.6.8: "In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. Wherever possible and after product-specific validation, the pyrogen test is replaced by the monocyte- activation test (2.6.30)."* In spite of this explicit instruction to replace the rabbit pyrogen test (RPT) by its *in vitro* alternative, the animal test continues to be widely used.

At its annual conference in 2018,[2] the European Partnership for Alternative Approaches to Animal Testing (EPAA) reported on a survey performed among European companies and testing institutes that still routinely perform the RPT and found that there is little incentive to perform alternative testing when a pyrogen test is prescribed in a monograph. The regulatory burden linked with the change to the *in vitro* test was also mentioned.

Reading the Ph. Eur. texts only, users reported a potential discrepancy between monographs and EU Directive 2010/63/EU:[3]

"Article 4

Principle of replacement, reduction and refinement

1. *Member States shall ensure that, wherever possible, a scientifically satisfactory method or testing strategy, not entailing the use of live animals, shall be used instead of a procedure."*

"Article 13

Choice of methods

1. *Without prejudice to national legislation prohibiting certain types of methods, Member States shall ensure that a procedure is not carried out if another method or testing strategy for obtaining the result sought, not entailing the use of a live animal, is recognised under the legislation of the Union."*

According to Article 13 of the directive, the instruction given in chapter 2.6.8 – to use an alternative to the animal test – should be applied systematically, but this is not done in practice.

In view of the situation, the complete removal of the RPT from the Ph. Eur. is necessary if the aim is to move towards the exclusive use of *in vitro* tests for the control of pyrogens.

Currently, chapter 2.6.8 is prescribed in 59 texts of the Ph. Eur.: three general monographs (including 2034 *Substances for pharmaceutical use*), three dosage form monographs (including *0520 Parenteral preparations*), three general chapters and 50 individual monographs, covering such diverse products as antibiotics, human vaccines and blood products. In June 2021, the Ph. Eur. Commission endorsed the strategy for the replacement of *2.6.8* in all of these 59 texts.[4] A new general chapter *5.1.13. Pyrogenicity* will be introduced in the Ph. Eur., which will provide guidance to help users decide on their own approach to pyrogenicity testing, based on a risk assessment: depending on the potential presence of non-endotoxin pyrogens, the user will have the choice between an *in vitro* pyrogen test or a test for bacterial endotoxins. Suppressed from all texts of the Ph. Eur., chapter 2.6.8 will no longer be an option and will ultimately be suppressed from the Ph. Eur. The whole exercise will take approximately 5 years and stakeholders will be consulted via the usual channels with, in 2023, the chance to consult all proposed revisions and the new general chapter *5.1.13* – currently under preparation – in Pharmeuropa online[5] and to comment as necessary.

Recombinant Factor C

The test for bacterial endotoxins uses, as its main reagent, the amoebocyte lysate from an animal, the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). Discussions among Ph. Eur. experts on the use of a synthetic alternative to this natural reagent, recombinant factor C (rFC), have been ongoing since 2006. It took over a decade to collect sufficient data for the method using the synthetic reagent to be described in the Ph. Eur. A major breakthrough came on July 1, 2020 with the publication of general chapter *2.6.32. Test for bacterial endotoxins using recombinant factor C* in the Ph. Eur.,[6] giving an official status to the procedure using the recombinant reagent. In January 2021, the procedure entered official use as a Ph. Eur. method. In April 2021, the EDQM broadcast a webinar on the bacterial endotoxin test using rFC, explaining its current status as an alternative to the bacterial endotoxin test using the amoebocyte lysate.[7]

General chapter 2.6.14. *Bacterial endotoxins* gives a choice of six methods, A to F (gel-clot method: limit test, gel-clot method: quantitative test, turbidimetric kinetic method, chromogenic kinetic method, chromogenic end-point method, or turbidimetric end-point method), the Ph. Eur.'s aim would be to add a seventh method, method G, that could be used instead of any of the other methods. However, because the chapter has undergone International Harmonisation within the Pharmacopeial Discussion Group (PDG), no changes can be made to the chapter without the agreement of the other participating pharmacopoeias (United States Pharmacopeia and the Japanese Pharmacopoeia).[8] The topic is currently under discussion within the PDG.

Animal Welfare

The question of animal welfare is often raised in the context of rFC. The Ph. Eur. approach to this issue is laid out in its Introduction: "***Use of animals***. *In accordance with the European Convention on the protection of animals used for experimental and other scientific purposes (1986), the Commission is committed to the reduction of animal usage wherever possible in pharmacopeial testing, and encourages those associated with its work to seek alternative procedures. An animal test is included in a monograph only if it has clearly been demonstrated that it is necessary to achieve satisfactory control for pharmacopeial purposes.*" Strictly speaking, rFC does not fall within the scope of the above-mentioned Council of Europe Convention, as the horseshoe crab is not directly used in pharmacopoeia testing. Nonetheless and very importantly, rFC avoids the use of a reagent extracted from a natural source and endangered species. As a single molecular entity, it also has higher standardization potential and as such represents significant technological progress. Last but not least, there is the crucial question of supply of the reagent: with horseshoe crabs absent from its coastlines, for Europe, the use of a recombinant alternative avoids potential supply shortages and a dependency on non-European countries; the potential supply concerns prompted by complete reliance on a single natural resource (the horseshoe crab) must also be taken into account. The recombinant source is an obvious step towards independence in this regard.

The pyrogenicity project fits perfectly within the scope of the "Replacement" aspect of the 3Rs, i.e. "technologies or approaches which directly replace or avoid the use of animals in experiments where they would otherwise have been used." Although the replacement of animals is a significant achievement in itself, there will be additional benefits from changing from *in vivo* to *in vitro* tests, including increased scope for standardization and reduced variability which, together, constitute a significant technological advancement. The situation will be reviewed in five years, after the respective texts have undergone their revision process.

Conclusion

Over the last 50 years the Ph. Eur. has addressed the question of pyrogenicity using the analytical techniques available at the time, moving from animal tests towards *in vitro* methods and therefore promoting the use of standardized methods for a better control of medicines in Europe. The Ph. Eur. has recently engaged on a path that will put an end to the use of rabbits in pyrogen testing and increase the use of synthetic reagents for the detection of bacterial endotoxins.[4]

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