Reduction, Replacement and Refinement of Animal Tests in the European Pharmacopoeia: Recent Developments for Monographs on Biological Substances and Preparations

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1. INTRODUCTION

Since the opening for signature of the European Convention for the Protection of Animals Used for Experimental and Other Scientific Purposes in 1986, the European Pharmacopoeia has carried out a programme of work committed to replacing, reducing and refining the use of animals for monograph requirements (Castle, 1998, Artiges, 1999). This has involved to a large extent monographs on biological substances; biological preparations, plasma products and antibiotics. Recent achievements have been reported (Charton, 1999a, 1999b). The present report outlines results over the last year.

2. BIOASSAYS

2.1. *Oxytocin, Calcitonin (salmon)*

Since 1 January 2000, HPLC assays are used officially in the monographs on *Calcitonin (salmon)* (0471), *Oxytocin* (0780) and *Oxytocin concentrated solution* (0779) as replacements of bioassays. The replacements were achieved as a result of collaborative studies organised by EDQM, involving world-wide manufacturers (Briançon, 1999a, 1999b).

2.2. *Tetracosactide*

The monograph on *Tetracosactide* (0644) includes an assay on isolated rat adrenal cells. The replacement of the biological assay by an HPLC assay is under study. At the moment information is being gathered on a suitable HPLC method. Readers are kindly requested to send suggestions to the Secretariat.

2.3. *Corticotropin*

The *Corticotropin* (0759) monograph includes an assay on isolated rat adrenal cells. It is questionable whether this substance is still used therapeutically. An enquiry published in *Pharmeuropa 12.3* is under way to find this out. If the substance is no longer used the monograph will no longer be needed.

2.4. *Erythropoietin concentrated solution*

As a result of a collaborative study organised by EDQM (Bristow and Charton, 1999), capillary zone electrophoresis (CZE) has proved to be suitable for the detection and quantification of EPO isoforms. It is envisaged that an *in-vitro* assay used in conjunction with CZE could lead to the replacement of the bioassay. The project is under study. At the moment information is being gathered on a suitable *in-vitro* assay. Readers are kindly requested to send suggestions to the Secretariat.

2.5. *Haemophilus type b conjugate vaccine*

In view of the improved methods for physicochemical and immunochemical characterisation of this vaccine, the assay in mice has now been removed from the monograph.

2.6. *Veterinary clostridial vaccines (novyi, perfringens, septicum)*

The potency test for vaccines is carried out by vaccination of rabbits followed by determination of serum antibody levels induced. The monograph has been revised to allow immunochemical determination of antibodies as an alternative to the toxin-neutralisation test, which uses large numbers of mice and causes a high degree of distress. A reference serum has been established within the Biological Standardisation Programme to facilitate introduction of the *in vitro* antibody determination.

2.7. *Swine erysipelas vaccine (inactivated)*

During revision of the monograph following discontinuation of the International Standard, the batch potency test using six groups of mice (three test, three reference) has been replaced by a test using two groups (one test, one reference) since this is sufficient for routine release of batches of vaccine. The test uses virulent challenge and work is being carried out in the Biological Standardisation Programme to validate a serological model for a future revision of the monograph.

3. BIOLOGICAL SAFETY TESTS

3.1. *Abnormal toxicity (2.6.9)*

Apart from streptokinase, the test for abnormal toxicity has now been removed as a requirement for routine application in monographs on biological substances.

3.2. *Histamine (2.6.10)*

The test for histamine has now been removed as a requirement for routine application in monographs on biological substances

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3.3. *Pyrogens (2.6.8)*

The pyrogen test is prescribed in the following monographs on biological substances: *Aprotinin* (0580), *Aprotinin concentrated solution* (0579), *Gonadotrophin (chorionic)* (0498), *Gonadotrophin (equine serum for veterinary use)* (0719), *Protamine sulphate* (0569), *Protamine hydrochloride* (0686), *Somatostatin* (0949), *Streptokinase* (0356), *Urokinase* (0695), *Urofollitropin* (0958). An enquiry was published in *Pharmeuropa 10.4* (December 1998) for the replacement of the pyrogen test by the test for bacterial endotoxins (2.6.14), requesting manufacturers to send the following information:

— method proposed as the official method (A, B, C, D or E, as described in Chapter 2.6.14 of the European Pharmacopoeia),

— validation data for application of the method in place of pyrogen test,

— evidence that use of the bacterial endotoxin test in place of the pyrogen test has been accepted by a regulatory authority,

— details of any problems or restrictions in applying the chosen method,

— proposal for a limit and data to support the limit.

Manufacturers submitted the corresponding files, which were later examined by European Pharmacopoeia specialists on the bacterial endotoxin test. Consensus limits were agreed on after discussions between specialists and manufacturers. Proposals for monograph revisions were then published in *Pharmeuropa 11.4* (December 1999). In June 2000, the European Pharmacopoeia Commission adopted the revised monographs on *Aprotinin*, *Aprotinin concentrated solution*, *Gonadotrophin (chorionic)*, *Gonadotrophin (equine serum for veterinary use)*, *Protamine sulphate*, *Protamine hydrochloride*, *Somatostatin*, *Urofollitropin*. The date of implementation of the revised monographs is 1 January 2002.

Monographs on *Streptokinase* and *Urokinase* are currently under revision for other purposes and the pyrogen test is therefore still in application. In the specific case of *Streptokinase*, which is produced by gram-positive bacteria, there is some concern that the substance may contain some non-limulus reactive pyrogens. The development of an *in-vitro* pyrogen test that could detect such pyrogens would facilitate the deletion of the pyrogen test in the monograph.

3.4. *Alternative to pyrogens*

The previous paragraph has shown the limits of the bacterial endotoxin test for some biological products. Among the other monographs prescribing the pyrogen test in the third edition of the European Pharmacopoeia, there are eight monographs on blood products, seven monographs on dosage forms, eight monographs on excipients and six monographs on vaccines. Each monograph needs to be examined individually to verify whether the pyrogen test can be replaced either by the LAL test or by an *in-vitro* pyrogen test. The European Pharmacopoeia has set up a Group of Experts to develop *in-vitro* alternative tests for pyrogen testing. It is foreseen that when a validated *in-vitro* pyrogen test is available, it will be included in the European Pharmacopoeia together with appropriate guidelines for validating the *in-vitro* pyrogen test as a replacement of the pyrogen test.

4. CONCLUSION

A summary of the work achieved and of the projects in progress is given in Tables 1 and 2. The replacement of animal tests by physicochemical tests has been achieved in most of the monographs on biological substances. Much work remains to be done for vaccines and blood products. Readers will remain informed on the progress made in the remaining projects.

5. AKNOWLEDGEMENTS

The authors wish to thank Dr. S. Poole and Dr. M. Gommer for their careful evaluation of the files submitted by manufacturers for the replacement of the pyrogen test by the LAL test following the *Pharmeuropa* enquiries.

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Table 1 — *Summary of the work achieved and current projects for biological substances*

Monograph	Animal test	Replaced by	Date of application
Aprotinin	Pyrogen test Histamine Abnormal toxicity	LAL Deleted (*) Deleted (*)	1 January 2002 1 January 2001 1 January 2001
Aprotinin conc. sol.	Pyrogen test Histamine Abnormal toxicity	LAL Deleted (*) Deleted (*)	1 January 2002 1 January 2001 1 January 2001
Calcitonin (salmon)	Bioassay	HPLC	1 January 2000
Chymotrypsin	Histamine	Deleted (*)	1 January 2001
Corticotropin and Corticotropin assay	Bioassay	Monograph and method to be deleted?	*Pharmeuropa 12.3(**)*
Erythropoietin conc. sol.	Bioassay	CZE + *in-vitro* assay	CZE : 1 January 2002 *in vitro* assay under study
Gonadotrophin chorionic	Pyrogen test	LAL	1 January 2002
Gonadotrophin equine serum for vet. use	Pyrogen test	LAL	1 January 2002
Oxytocin	Bioassay	HPLC	1 January 2000
Oxytocin conc. sol.	Bioassay	HPLC	1 January 2000
Protamine sulphate	Pyrogen test Abnormal toxicity	LAL Deleted (*)	1 January 2002 1 January 2001
Protamine HCl	Pyrogen test Abnormal toxicity	LAL Deleted (*)	1 January 2002 1 January 2001
Somatostatin	Pyrogen test	LAL	1 January 2002
Streptokinase	Pyrogen test	LAL	*Pharmeuropa* 11.4(**)
Tetracosactide	Bioassay	HPLC	Under study
Urofollitropin	Pyrogen test	LAL	1 January 2002
Urokinase	Pyrogen test	LAL	*Pharmeuropa* 11.4(**)
Trypsin	Histamine	Deleted (*)	1 January 2001

(*) Deleted from the Tests section and moved to the Production section.
(**) Enquiry in progress.

Table 2 — *Summary of the work achieved and current projects for vaccines*

Monograph	Animal test	Modification	Date of application
Haemophilus type b conjugate vaccine	Assay	Deleted since physico-chemical characterisation is now sufficient	1.9.2000
Clostridial vaccines novyi, perfringens, septicum) (veterinary)	Potency	*In vitro* determination of antibody allowed as an alternative to the toxin-neutralisation test in mice	1.1.2001
Swine erysipelas vaccine (inactivated)	Batch potency	Number of groups of mice reduced from 6 to 2 Serological model under study	1.1.2001
Tetanus vaccines (human/veterinary)	Assay/Potency	Serological model under study	