3.0 Substances Used for the Validation of *In Vitro* Pyrogen Test Methods

3.1 Rationale for the Substances or Products Selected for Testing

A validation study should evaluate an adequate subset of substances and product types that are to be tested by the proposed test method. In response to a request for additional information, the rationale for the specific test substances selected for inclusion in the validation studies was provided by ECVAM, which included stability of the endotoxin-spike, relevance, availability/feasibility, and cost (see **Appendix C**). Briefly, to maintain the desired concentration of the endotoxin-spike solution over the time period needed for the validation studies, the test substances and the endotoxin-spike solution were provided separately to the test laboratories and mixed prior to testing. As for relevance, only substances intended for i.v. injection were selected. In addition, test substances consisted solely of marketed parenteral pharmaceuticals that were labeled as free from detectable pyrogens such that these data were available for comparison to the validation study results.

3.2 Number of Substances

A total of 13 substances were included in the performance analysis of each of the five *in vitro* test methods. Ten substances, each spiked with four different concentrations of endotoxin (0, 0.25, 0.5, and 1.0 EU/mL, with 0.5 EU/mL tested in duplicate), were used to evaluate accuracy. Three substances, each spiked with three concentrations of endotoxin (0, 0.5, and 1.0 EU/mL, with 0 EU/mL tested in duplicate), were used to assess intralaboratory reproducibility.

3.3 Identification and Description of Substances Tested

As indicated in **Section 3.1**, the test substances selected for use in the validation studies were marketed parenteral pharmaceuticals. **Table 3-1** lists the 10 test substances used to evaluate accuracy, and **Table 3-2** lists the three test substances used to evaluate reproducibility. In response to a request for additional information, ECVAM provided the lot numbers of the substances used in accuracy evaluation for the validation study, which demonstrated that they were identical (**Appendix C**). However, some of the lots tested in the catch-up validation study for the Cryo WB/IL-1β test method were different (i.e., Fenistil and Sostril) because the original lots were no longer available. One test substance (i.e., Orasthin) was no longer available and was replaced with Syntocinon, which contains the same active ingredient.

Table 3-1 Parenteral Drugs Used in the Validation Studies for Determining Test Method Accuracy[1]

Test Substance[2]	Source	Lot Number(s)	Active Ingredient	Indication	MVD (-fold)
Beloc®	Astra Zeneca	DA419A1	Metoprolol tartrate	Heart dysfunction	140
Binotal®	Grünenthal	117EL2	Ampicillin	Antibiotic	140
Ethanol 95%	B. Braun	2465Z01	Ethanol	Diluent	35
Fenistil®	Novartis	21402 26803[3]	Dimetindenmaleat	Antiallergic	175
Glucose 5%	Eifelfango	1162 3132[3]	Glucose	Nutrition	70
MCP®	Hexal	21JX22	Metoclopramid	Antiemetic	350
Orasthin®	Hoechst	W015	Oxytocin	Initiation of delivery	700
Sostril®	Glaxo Wellcome	1L585B 3H01N[3]	Ranitidine	Antiacidic	140
Syntocinon®	Novartis	S00400	Oxytocin	Induction of labor	-
Drug A - 0.9%NaCl	-	-	0.9% NaCl	-	35
Drug B - 0.9% NaCl	-	-	0.9% NaCl	-	70

Abbreviations: MVD = Maximum valid dilution
[1]Each substance was tested in all five *in vitro* pyrogen test methods.
[2]Each test substance was spiked with 0, 0.25, 0.5, or 1.0 endotoxin units/mL (EU/mL) of endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]), with 0.5 EU/mL tested in duplicate. Each sample contained the appropriate spike concentration when tested at its MVD.
[3]Indicates the lot number used in the catch-up validation study for the Cryopreserved Whole Blood/Interleukin-1β test method.

Table 3-2 Parenteral Drugs Used in the Validation Studies for Determining Test Method Reproducibility[1]

Test Substance[2]	Source	Agent	Indication
Gelafundin®	Braun Melsungen	Gelatin	Transfusion
Haemate®	Aventis	Factor VIII	Hemophilia
Jonosteril®	Fresenius	Electrolytes	Infusion

[1]Each substance was tested in all five *in vitro* pyrogen test methods.
[2]Each test substance was spiked with 0, 0.5, or 1.0 endotoxin units/mL (EU/mL) of endotoxin (WHO-LPS 94/580 [*E. coli* O113:H10:K-]), with 0 EU/mL tested in duplicate. Each sample contained the appropriate spike concentration when tested at its maximum valid dilution.

3.4 Sample Coding Procedure

According to the ECVAM BRDs (Section 3.4), the 10 test substances and the four spike concentrations used for the evaluation of accuracy were blinded to the testing laboratories. For the reproducibility analyses, although the three spike concentrations were blinded to the participating laboratories, the identities of the three test substances were not.

3.5 Rationale for the Selection of the Recommended Reference Substances

Reference substances are used to assess the accuracy and reliability of a proposed, mechanistically and functionally similar test method and are a representative subset of those used to demonstrate the reliability and accuracy of the validated reference test method (in this case, the RPT). These substances should:

- Represent the range of responses that the validated test method is capable of measuring or predicting
- Have produced consistent results in the validated test method
- Produce responses that reflect the accuracy of the validated test method
- Have well-defined chemical structures and/or compositions
- Be readily available
- Not be associated with excessive hazard or prohibitive disposal costs

For evaluating test method performance, each of the test substances used in the ECVAM validation studies was spiked with a Gram-negative endotoxin standard (WHO-LPS 94/580 [*E. coli* O113:H10:K-]). Two different sources of endotoxin (i.e., *E. coli* EC-5 and *E. coli* EC-6), which were reported to be identical to the WHO standard, were used in the validation studies (Hochstein et al. 1994; Hoffman et al. 2005a). Endotoxin was selected as a "model" pyrogen for inclusion based on its availability in a standardized form and because of the known ability of monocytic cells to respond to endotoxin-based pyrogens. Endotoxin was also used as a positive control and for qualifying the *in vitro* test methods during interference testing. It is also used when performing the BET. As described in **Section 4.0**, the response of the reference test method (i.e., RPT) to endotoxin is well documented. For this reason, the threshold pyrogen dose used for establishing the decision criteria for the *in vitro* test methods was based on historical RPT data. Importantly, no other non-endotoxin-based pyrogenic substances are presently available in a standardized form.