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ROUTINE PYROGEN TESTING

BY K. L. SMITH

From the Bioassay Division, Standards Department, Boots Pure Drug Co., Ltd., Nottingham

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THE British Pharmacopœia specifies that aureomycin, streptomycin, penicillin, heparin and injection of heparin, injection of calcium gluconate and water for injection should be substantially free from pyrogens and indicates that such freedom may be established by showing that their intravenous injection into rabbits is not followed by a marked increase in body temperature. The doses to be administered are specified. The injections are required to be made into 3 rabbits and the mean maximum temperature rise in the rabbits during the 3 hours following injection may not exceed the pre-injection temperatures by more than 0·6° C. Table I shows the doses prescribed by the British Pharmacopœia.

It would be expected that the application of this test to these substances even from large-scale manufacture would produce little difficulty and this is true in the case of the antibiotics, heparin and calcium gluconate, but some difficulties do arise in the case of water for injection.

The British Pharmacopœia includes a monograph on water for injection

and specifies that it should be used in the manufacture of certain preparations intended for injection and gives precise instructions for its preparation.

"Distil potable water in a neutral or metal still fitted with an efficient device to prevent entrainment. Reject the first portion of the water and collect the remainder in a neutral glass container. Immediately sterilise by heating in an autoclave."

TABLE I

REQUIREMENTS OF THE B.P. TEST FOR PYROGENS

Preparation	Dose per kg. of body weight	Maximum mean temperature rise permitted in 3 rabbits
Aureomycin	5000 units	0·6° C.
Streptomycin ..	2000 units	
Penicillin	2000 units	
Heparin	2000 units	
Calcium gluconate ..	200 mg.	
Water for injection ..	10 ml.	

We have a strong suspicion that it is the official intention to prescribe procedures suitable for the dispensing pharmacist and to leave manufacturers to develop their own methods for producing materials of equal quality. Even so we would suggest that the instructions be improved still more by indicating that some effort should be made to render the container pyrogen-free especially, as must frequently be the case, if the test for freedom from pyrogens is omitted.

A manufacturer will wish to produce water for injection both for sale as such and for the manufacture of other preparations. In the case of that required for sale he can, in so far as the larger packs, such as 1 l. and 0·5 l., are concerned, follow the method prescribed and fill straight from the still. When smaller volumes are desired he may find it more convenient to fill into large containers which are not themselves suitable for autoclaving and thence fill into the smaller packs. In the case of water intended for the manufacture of preparations for injection, the volume required will certainly make it impracticable to use the official method of collection and sterilisation and the manufacturing processes will have to be carried out on the assumption that the water is pyrogen-free, a procedure which will, of course, necessitate the testing of the final preparation where this is possible.

This principle has much to commend it. In fact we have always considered that the official requirements were a little inadequate in inferring that it is sufficient to use pyrogen-free water to prepare solutions intended for injection when the other ingredients are not so tested. Many of the B.P. preparations are not, however, in their final form, amenable to pyrogen tests and injection of insulin is an example of this. Since in the scheme of testing which we have adopted the water delivered by the still is examined, either directly or indirectly, more than once in any day it is

considered that the suitability of the water for such preprations can be assumed with confidence.

The conditions of our examinations do deviate from the official instructions, and before proceeding further these points of difference should be mentioned.

1. *The volume to be injected.*

This is specified as 10 ml./kg. It is our practice to withdraw the plunger of a 25 ml. all-glass syringe, to charge the syringe with as great a volume as it is capable of holding and to inject this quantity, which is roughly 30 ml., irrespective of the rabbit weight, which, in our colony may be as great as 3·5 kg. We are satisfied that this method is not open to great criticism since in many cases the rabbits receive a larger volume than is officially prescribed and since it is general experience that the slope of the line relating temperature rise to log. dose is very shallow so that a small change in dose has little effect on the level of the response.

We were somewhat at a loss to decide what examination should be made on small volume ampoules of water for injection and finally adopted the procedure of injecting into the animal the total contents of the ampoule or 25 ml. plus, whichever is the smaller.

2. *It is suggested that water be warmed to* 37° *C. before injection.*

It would be splitting hairs to suggest that, as the temperature of a rabbit is about 39·5° C., that temperature should have been the one chosen and in any case we have ourselves long abandoned the practice of preheating the water. We are satisfied that the injection of 25 ml. of distilled water at room temperature has no noticeable effect on the recorded temperature of the rabbit. We therefore make the injection at room temperature.

3. *The number of rabbits specified is* 3, *the critical temperature is* 0·6° *C.*

We take only 2 rabbits for a test and pass if the rises of both are below 0·6° C. and the mean less than 0·5° C.; if this is not so a further 2 rabbits are injected and the sample is considered satisfactory if the mean of all 4 is less than 0·6° C.

4. *Use of isotonic solutions.*

The British Pharmacopœia also offers for consideration the suggestion that the injection be made isotonic by means of pyrogen-free sodium chloride. We have decided against this following our policy of reducing variables to a minimum. Thus in keeping with this decision not to add sodium chloride when examining water for injection, we always consider that the volume of solutions should be kept to a minimum when examinations of the antibiotics or heparin are made.

Similar examinations are made of all other solutions intended for intravenous injection where the volume packed exceeds 10 ml. unless a test is specifically called for, e.g. heparin and calcium gluconate.

Some consideration at this stage should be given to our conception of a batch. We insist that this should be characterised by some common treatment. A batch of water in 1 l. or 0·5 l. packs is that which has been filled consecutively in a limited time and is sterilised at one autoclaving. Similarly a batch of saline solution or glucose-saline solution is determined by the volume of the container in which the solution is prepared (this is roughly 300 l.) and, of course, must be sterilised at one autoclaving. Ampoule packs of distilled water again come in for special treatment. In this case the batches are related to filling periods which correspond to the intervals between the lunch, dinner and tea-breaks a division which also provides a convenient lot size for sterilisation at one autoclaving.

Manipulative Procedures

Temperature.

The temperature usually recorded is that obtained per rectum and the simplest method is obviously that using clinical thermometers. Some objection to such a method has been raised by Dare[1] in that when rabbits are restrained the use of clinical thermometers gives misleading results. For many years it was our practice to use "half-minute" clinical thermometers inserted 3 inches and left in position for 2 minutes before the temperature was read. We found it unnecessary to restrain the animals which were nursed on the operator's lap and are quite satisfied that this method gives results parallel to the thermocouple methods.

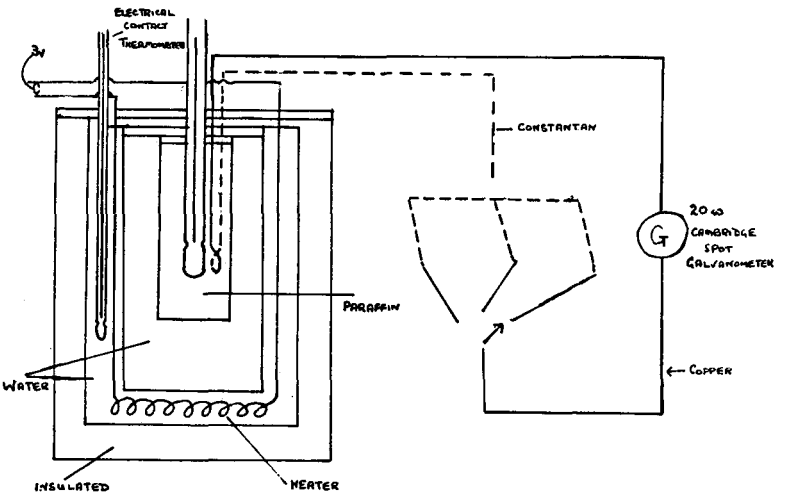

DIAGRAM OF THERMOCOUPLE UNIT

FIG. 1.

The thermocouple technique is, however, more convenient and for routine work it is present practice to use copper-constantan thermocouples. The arrangement we use, is a copy of that in use at the Wellcome Foundation, Ltd., before they improved on it still further by including a recording galvanometer. It is shown diagramatically in Figure 1. A constant

reference temperature is obtained by the use of the apparatus shown on the left of Figure 1. This consists essentially of 4 concentric containers. The outer pack is an insulated jacket, the next carries a low-tension heater immersed in water together with its thermostatic control. Internal to this is a further container of water and in the centre a container filled with liquid paraffin in which the reference thermocouple is placed. It is our experience that the temperature of the innermost container is constant as determined by a Beckman thermometer.

The wire we use for the thermocouples was specially prepared for us by Messrs. Duratube[2]. It consists of copper and constantan wire (26 S.W.G.) covered separately with polyvinyl chloride and twisted together and covered with a further polyvinyl chloride sheath. A suitable length is cut off the wires exposed by slitting down the sheath, a junction made by twisting and soldering and the sheath replaced and sealed by means of a soldering iron.

A common 10-ft. length of wire is used for the reference thermocouple, each recording thermocouple is 4 ft. 6 in. long and is switched into circuit by means of a wafer radio switch in the copper side of the circuit, in which side is also inserted the 20 ohm Cambridge spot galvanometer, the scale of which has been recalibrated in ° C.

Under the conditions used the apparatus works extremely well, we have contrived to have all the dissimilar junctions assembled as closely as possible so that any change in ambient temperature will affect all junctions equally. The error likely to arise if this is not attended to was made evident when we attempted to extend the apparatus.

The use of thermistors, which possess a high negative temperature coefficient of resistance, for the determination of temperature in the aural cavity of man has been described[3], and we have examined the possibility of applying this method in our laboratory to determine the rectal temperatures in rabbits. On theoretical grounds there is much to commend them. The need for a reference temperature is dispensed with, the error due to ambient temperature on junctions is removed and it is possible to measure the temperature at greatly removed distances. Difficulties have arisen in their adaptation, for example, the characteristics of each thermistor vary greatly and call for special calibration, but these have been overcome with the help of a skilled electronic engineer.

In a commercial laboratory certain economic if non-academic issues arise in considering these methods of determining temperature.

A clinical thermometer if broken is easily replaced. The broken thermocouple is also easily repaired, but the effect of a power breakdown is important. It is for that reason that we have used a low-tension heater in our reference temperature set-up. This normally derives its power from a mains supply, but in the event of a power breakdown it is automatically switched to a storage battery. We have decided that when our thermistor unit is in full use the valve voltmeter will be battery operated and duplicated. Even so we shall be highly dependant on help outside our own laboratory when faults need to be repaired.

Rabbits.

In general the rabbits used in our laboratory are the Belgian hare type of both sexes. These are purchased from dealers and the official instructions are followed in that they are not used until their weight exceeds 2·5 kg. They are discarded when their weight reaches 4·0 kg. The only other restriction made is to use rabbits only if their initial temperature is between 38·8 and 39·8°C. The colony consists of 100 usable rabbits which are brought into the testing unit in groups of 20. They are brought into the laboratory in sequence, but the treatments on any day are randomised. We do not discard rabbits which have responded pyrogenically as has been suggested, but do keep a record of the responses of each rabbit, to check whether an erratic low response could be attributed to previous treatment.

Restraining boxes.

When methods are employed which call for the thermocouple to be inserted for 4½ hours or so some means of restraining the animals with a minimum of discomfort must be sought. Each worker will have his own pet method and may discount all others. That used by us was designed to allow the rabbit to sit in a normal position with the neck in a stock and was more or less built around a sitting rabbit.

Racks.

Figure 2 shows these boxes and also the rack on which the rabbits are kept for the duration of the test. It shows also the reference temperature unit and the control panel.

Test site.

In the days when clinical thermometers were used the tests were conducted in a large and congested laboratory with a fair amount of traffic in and out and were accompanied by few erratic results. When the unit shown was erected the number of erratic results increased. Removal of the apparatus to a smaller and quieter room reduced the number of erratic results to such an extent that we now consider minimum disturbance during the test to be essential.

Syringes and glassware.

It would be ridiculous to mar the test by introducing pyrogens in its conduct, and we consider it essential to use a separate syringe and needle for each animal and to submit these and flasks in which solutions are prepared to oven treatment to render them pyrogen-free. Welch *et al.*[4] have indicated that heating to 250° C. for 40 minutes is a suitable treatment. We have no reason to suspect our own procedure of heating to 200° C. for 2 hours plus and since this treatment appears adequate it has never seemed worth while to determine the minimum safe time.

Conduct of the test.

In carrying out the test the rabbits are placed in their boxes and the thermocouples inserted. They are inserted to a distance of 4 inches and

FIG. 2.

secured by tying to the tail with a piece of string. Commencing one half-hour later the temperatures are recorded at 20-minute intervals throughout the remainder of the test even whilst the injections are being made. The injection time is noted and the mean of 3 temperatures taken before this, is regarded as the preinjection temperature and the highest temperature in the 3 hours after injection taken as final temperature.

The results that have been obtained in our examinations of 1 l. and 0·5 l. packs of water for injection, glucose-saline solution and normal saline solution are shown in Table II, which illustrates the distribution of

the temperature rises which have been obtained during the examinations and shows the number of batches passed and failed. It should be added that the highest mean rise recorded in this group was 0·92° C., but mean rises of 1·0° C. and above are readily obtained with preparations known to be pyrogenic.

TABLE II

DISTRIBUTION OF TEMPERATURE RISES DURING PYROGEN TESTS ON 1 l. AND 0·5 l. PACKS

Preparation	°C → / RABBITS ↓	0·00 – 0·09	0·10 – 0·19	0·20 – 0·29	0·30 – 0·39	0·40 – 0·49	0·50 – 0·59	> 0·6
Water for injection	2	2	5	9	1	5	—	
	4				1	6	4	1
		← 33 →						1
Normal saline	2	21	72	69	59	28	—	
	4			5	8	12	8	6
		← 282 →						6
Glucose saline	2	8	15	21	16	11	—	
	4				5	6	2	
		← 84 →						0
	Sum	← 399 →						7
		← Pass →						← Fail →

It should be recorded also that of the seven batches failed under the tests described in Table II, the 4 normal saline solutions which have been retested after storage for 3 months now pass the official test quite easily. This agrees with the observations made by Paris and Collier.[5]

SUMMARY

1. The examination of preparations from large-scale manufacture for freedom from pyrogens is described.

2. Typical results from the examination of 1 l. and 0·5 l. packs of water for injection, glucose-saline and solution and normal saline solution are presented.

3. Evidence supporting the claim that reduction in the pyrogen levels take place on storage is provided.

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