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SCIENCE PAPERS AND DISCUSSIONS

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THE STERILISATION, STABILITY AND TOXICITY OF CONGO RED INJECTIONS

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CONGO red consists mainly of the disodium salt of 4:4'-bis-(1-amino-4 sulpho-2-naphthaleneazo)diphenyl and it usually contains traces of sodium chloride. It is chiefly used in an 0·5 to 1·5 per cent. solution for the detection of amyloidosis.

There have been conflicting reports on the toxicity of the dye, on methods of preparing the injection and on its stability in solution[1–7]. Congo red is recognised as being a rather variable product and the British Pharmaceutical Codex 1954 includes biological tests for freedom from undue toxicity and for the absence of pyrogens. There is, however, no statement about limits of pH value. The United States Pharmacopeia XV gives the pH of a solution of Congo red (no strength stated) as 8 to 9·5 and states that solutions decompose on exposure to acid fumes. Injection of Congo Red U.S.P. is required to have a pH between 7·0 and 9·0.

The United States Foods and Drugs administration[8] has stated that "in the preparation of solutions of Congo red it is important that no free colour acid should be present. The presence of free colour acid may be due to exposure of the solid or of solutions to acid".

While large numbers of injections of Congo red have been made without toxic symptoms[9–18], there have been reports of untoward reactions such as abdominal pain, palpitation and rigors[19,20], and a few fatalities have occurred[21]. Some of these have been attributed to anaphylactic reactions in previously sensitised people.

For the preparation of Congo red solutions for injection the British Pharmaceutial Codex 1949 suggests that the dye should be dissolved in Water for Injection immediately before use. The Extra Pharmacopœia (Vol. I, 1941) states that such solutions should be used as soon as possible since the dye slowly hydrolyses in solution. However, the availability of commercially prepared solutions and the results of Richardson and Dillon[2] suggest that the dye is more stable in solution than is

generally supposed. Richardson and Dillon[2] prepared solutions containing 1 per cent. of Congo red with 5 per cent. of dextrose and found that they were stable almost indefinitely at 4° C. Solutions in physiological saline were only stable for 24 hours due to a salting out effect.

Wallace[5], who first described the use of the Congo red test in this country, prepared the injection by dissolving the dye in hot water, filtering through fine filter paper and boiling. He stressed the need for using specially purified dye and stated that solutions should be used within 12 hours or rigors may occur. He considered reactions were probably due to the use of concentrations over 1 per cent.[22]. Others have injected 1·5 per cent. solutions without ill effects[6].

In view of these conflicting statements we have investigated the effects of different methods of sterilisation, of pH changes and storage conditions on the toxicity of Congo red solutions. A preliminary note of our results was read at the General Assembly of the International Pharmaceutical Federation in 1953, leading to the adoption by the British Pharmaceutical Codex 1954 of autoclaving or filtration as methods for sterilising injection solutions[23].

Experimental and Results

Materials

The same sample of Congo red powder was used throughout the investigations except where otherwise stated, and was a commercial product intended for injection. The dye had been biologically tested by the manufacturers. Commercial solutions were obtained on the open market.

Methods

Preliminary experiments indicated that a 1 per cent. solution was suitable for the toxicity tests. Solutions were prepared by dissolving the dye in Water for Injection with the aid of heat and filtering through a No. 3 sintered glass funnel. The solutions were then submitted to different sterilisation and storage procedures. The samples were tested for acute toxicity by the intravenous route in mice. Male albino mice, weighing between 16 and 24 g., were randomised into groups of 10 mice and respective groups were injected at ascending dose levels. Mortalities were observed over 48 hours from which the LD50 doses and their limits of error were calculated[24].

Tests for the absence of pyrogens were carried out in rabbits by the method described in the British Pharmaceutical Codex 1954.

Effect of autoclaving, steaming and filtration. Part of a solution of Congo red was transferred to 10 ml. ampoules, half of which were autoclaved at 115° C. for 30 minutes and the rest steamed at 90 to 100° C. for 30 minutes. The remainder of the solution was filtered through a 5/3 bacteria-proof sintered glass funnel and the filtrate distributed aseptically into 10 ml. ampoules. These solutions were tested for toxicity: the results obtained are shown in Table I. There was no significant difference in the toxicities of the solutions subjected to the different processes.

Effect of Storage. A fresh solution of Congo red was prepared, distributed into three groups of ampoules and treated by the above methods. Half the number of ampoules in each group was stored at 4° C. and the remainder at room temperature, over a period of three months. The physical appearance of these solutions and their toxicities after storage were compared with a freshly prepared solution. The results are shown in Table II.

TABLE I

INTRAVENOUS TOXICITY IN MICE OF 1 PER CENT. CONGO RED SOLUTIONS

Solution	LD50 ml./20 g.	Limits of error (P = 0·95) per cent.
1. Autoclaved	0·75	76 to 132
2. Steamed	0·60	90 to 113
3. Filtered	0·63	94 to 106

The samples stored at 4° C. all showed a slight deposit, this being greatest in the samples sterilised by filtration. The deposit redissolved on warming and did not reappear on cooling to room temperature. The toxicity results show that the autoclaved and steamed solutions had not increased in toxicity during storage. The bacteriologically filtered solutions were much more toxic than the others, an observation which we found difficult to explain. We repeated

TABLE II

INTRAVENOUS TOXICITY IN MICE OF 1 PER CENT. CONGO RED SOLUTIONS STORED FOR THREE MONTHS

Solution	Appearance	LD50 ml./20 g.	Limits of error (P = 0·95) per cent.
1. Control; freshly prepared solution	Bright and clear	0·69	97 to 103
2. Autoclaved and stored at room temperature	Bright and clear. Slight deposit on inverting ampoule in bright light	0·69	97 to 103
3. Autoclaved and stored at 4° C.	Bright and clear, but considerable deposit on inverting ampoule in bright light	0·75	97 to 103
4. Steamed and stored at room temperature	Bright and clear. Slight deposit on inverting ampoule in bright light	0·66	94 to 106
5. Steamed and stored at 4° C.	Bright and clear, but considerable deposit on inverting ampoule in bright light	0·64	94 to 106
6. Filtered and stored at room temperature	Bright and clear, no deposit	0·48	95 to 105
7. Filtered and stored at 4° C.	Copious deposit	0·47	97 to 103

The deposits were readily soluble on warming and shaking the ampoules; the resulting solutions appeared and remained bright and clear.

the filtration method with a new solution and this time no increase in toxicity occurred during storage.

Ampoules remaining from the batch subjected to filtration and used in the earlier tests (solution 3 in Table I), when tested for toxicity after seven months' storage at room temperature, had an LD50 of 0·62 ml./20 g. mouse. Thus no increase in toxicity had occurred. The single occurrence of a solution with a high toxicity after filtration could not be ignored

and we investigated the possible causes further. In the preparation of the toxic solution filtration was prolonged. The solution took four hours to pass through the filter and the sterile solution was left overnight in a sterile sealed flask. The following morning some deposit was observed in the filtrate but this readily disappeared on shaking before the solution was transferred aseptically to sterile ampoules. Possible causes of the increased toxicity may have been oxidation, the effect of carbon dioxide during prolonged exposure to air, or contamination with acid from an imperfectly cleaned filter. These were investigated in turn.

Effect of prolonged exposure to air. A fresh solution was prepared, filtered through a 5/3 sintered glass funnel and the sterile filtrate kept in the flask overnight as before.

The LD50 of this solution was 0·61 ml./20 g. mouse with limits of error from 95 to 105 per cent. No increase in toxicity had therefore occurred. In another experiment the filtered solution was kept in a partly filled sterile 500 ml. infusion bottle for seven days with occasional thorough shaking. This solution also did not increase in toxicity. After storage at 37° C. for three months, in partly filled 10 ml. ampoules, the toxicity remained the same. Exposure to air could not have been the cause of the increased toxicity in our stored solutions after filtration, so we turned our attention to pH. Unfortunately the pH of our original toxic solution had not been taken and no more was available, so a fresh solution had to be prepared.

Effect of pH. The pH of a freshly prepared 1 per cent. solution of our sample of Congo red, measured electrometrically, was 10·1 and did not change during autoclaving. This was higher than the upper limit stated in the U.S.P. XV. The pH of a commercially prepared injection solution was 9·8 and of a 1 per cent. solution of another commercial powder intended for injection was 9·4. None of these samples, therefore, complied with the U.S.P. requirements.

To study the effects of increased acidity, graded amounts of 0·1N hydrochloric acid were added from a microburette to 10 ml. quantities of a freshly prepared Congo red solution. The pH values and the appearance of each solution were recorded and the toxicity determined. The results are shown in Table III.

Immediately a drop of acid was added to the dye solution a bluish-black precipitate appeared, but this readily dissolved on shaking to leave the solution bright red and clear down to pH 7. Any further increase in acidity caused a darkening in colour and the development of an opacity. Below a pH of 6·5 the solution changed to a purple colour and solid material was precipitated. These changes affected the toxicity of the solutions. Below pH 6·8 there was an increase in toxicity and at pH 6·5 solutions which contained solid material were immediately lethal to mice. The change in pH is therefore a probable explanation of the higher toxicity of the solution sterilised by filtration.

Toxicity of a solution of Congo red from another hospital. We also had the opportunity of examining some ampoules of a 1 per cent. solution of Congo red prepared in another hospital. The dye, from a different

TABLE III

EFFECT OF ADDING ACID TO CONGO RED SOLUTION

Ml. of 0·1 N HCl added to 10 ml. of 1 per cent. solution	Appearance of solution	pH	Mortality of mice
None	Blood red colour. Bright and clear. No precipitate	9·7	9/20
0·01	Blood red colour. Bright and clear. No precipitate	9·7	4/10
0·20	Blood red colour. Bright and clear. No precipitate	7·2	1/10
0·22	Blood red colour. Bright and clear. No precipitate	6·8	5/10
0·24	Blood red colour. Bright and clear. No precipitate	6·8	5/10
0·30	Dark red. Opaque. Trace of precipitate appeared on standing	6·5	3/3 immediately
0·40	Dark brownish red colour with trace of precipitate, becoming reddish violet with marked precipitate on standing	6·5	5/5 immediately
0·50	Brown colour with marked precipitate becoming violet with a thick deposit on standing	6·4	2/2 immediately

The mice were all given a dose of 0·64 ml. of 1 per cent. solution each, by intravenous injection. This represents the approximate LD50. Except where otherwise stated the mortalities were observed over 24 hours.

source than ours, was reported to give a dense precipitate when the solution was autoclaved.

The LD50 of this solution was 0·38 ml./20 g. mouse with limits of error from 90 to 110 per cent. The solution was much more toxic than usual. A fresh solution of the original solid material had an LD50 of 0·52 ml./20 g. mouse with limits of error from 95 to 105 per cent. We conclude that this was a bad batch of Congo red.

TABLE IV

PYROGEN TEST ON 1 PER CENT. CONGO RED SOLUTIONS

Solution	Mean maximum rise in body temperature in 3 rabbits
1. Autoclaved sample of our Congo red (sample 2, Table II)	0·11° C.
2. Commercial solution	0·34° C.
3. Solution from another hospital	0·42° C.
All solutions comply with the B.P.C. test.	

Effect of incompletely dissolved dye. Solutions of Congo red may be prepared extemporaneously by the addition of Water for Injection to an ampoule containing a weighed amount of Congo red. Without filtration there must be a danger of injection of solid material due to incomplete solution which is not easy to detect. With one sample of Congo red it took several minutes of vigorous shaking before the solution became bright and clear. Before solution occurred the injections were highly toxic to mice.

Effect of sodium chloride. A solution of Congo red in physiological saline was found to be much more toxic than an aqueous solution, confirming the findings of Richardson and Dillon[2].

Toxicity of a commercially prepared solution. We have examined the toxicity of a prepared solution of 1 per cent. Congo red for intravenous

use, made by the same firm who supplied our solid material. This sample had been stored at room temperature for at least six months. The pH of this solution was 9·8 and its LD50 was 0·74 ml./20 g. mouse, so its toxicity was the same as that of our own preparations.

Test for pyrogenicity. Examination of the three samples recorded in Table IV showed them to be within the limits for pyrogenicity prescribed by the British Pharmaceutical Codex 1954.

Conclusions

While solutions of Congo red can be prepared extemporaneously by the addition of water for injection to the solid powder, there is a danger of incomplete solution of the dye which is difficult to detect.

Intravenous injection of the solid dye may cause immediate death, so a filtered and sterilised solution of Congo red is to be preferred. We have described how such a solution may be prepared and we have shown that solutions of Congo red may be safely autoclaved, steamed or subjected to bacterial filtration.

Such solutions are stable for at least seven months at room temperature. Storage in a refrigerator is not recommended because the dye may precipitate and become difficult to redissolve. In the preparation of the solution there is no danger in prolonged exposure to the air, provided evaporation does not take place, but care must be taken to avoid contamination with acid. A real source of danger is incomplete washing of sintered glass filters.

Solutions with a pH less than 6·8 are opaque and dark red in colour. They are also highly toxic. We recommend that solutions for injection should be bright red and clear and have a lower limit of pH 7·0 as stipulated by the U.S.P.

The inclusion of sodium chloride in Congo red solutions is contraindicated for the toxicity is increased and the solutions are unstable. Solutions made with dextrose if autoclaved might also become dangerous as a result of a fall in the pH.

Summary

1. A method is described for the preparation of solutions of Congo red for injection.

2. Solutions may be autoclaved, steamed or filtered through a bacteria-proof filter.

3. Solutions are stable at room temperature for at least seven months and storage in a refrigerator is not recommended.

4. Prolonged exposure to air does not increase the toxicity provided evaporation does not take place.

5. The pH of the solution is very important. Acid solutions are dangerously toxic and we recommend the adoption of a lower limit of pH 7·0, as directed by the United States Pharmacopeia.

6. The addition of sodium chloride increases the toxicity of the solutions which may be unstable.

7. No solution of Congo red should be injected which is not bright red and clear.

8. The pyrogenicity of three different samples of Congo red was within the limits set by the British Pharmaceutical Codex, 1954.

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DISCUSSION

The paper was presented by MR. T. D. WHITTET.

DR. F. HARTLEY (London) said that different batches of Congo red might vary considerably in toxicity. It would be interesting to know whether the so-called "pyrogenicity" of different batches was a proper use of that term, as it seemed doubtful that bacterial pyrogenic contamination was the cause. One was a little puzzled as to how the authors reached their conclusion that in a particular batch of ampoules which were alleged to be toxic, this was due to the dye.

MR. K. L. SMITH (Nottingham) asked whether the suitability of Congo red could be established by physical characteristics. Would the sample used have passed the B.P.C. test for toxicity?

MISS M. H. NEAL (Kuala Lumpur) asked whether, in view of the somewhat short period for which the authors recommended the solution should be kept, it might not be advisable to make some labelling recommendations. It would seem that no storage tests were conducted at tropical temperatures. She pleaded with workers to put on storage tests at 34 to 37° C. and to record those results for the benefit of those who worked overseas.

MR. S. G. E. STEVENS (London) asked at what point the authors would anticipate toxic reactions if evaporation were conducted under aseptic conditions.

MR. T. D. WHITTET, in reply, agreed with Dr. Hartley that the toxicity was not the result of contamination with pyrogens. The reason for the B.P.C. test for pyrogens was probably due to the fact there had been complaints of unpleasant reactions, including rigors. Only one sample was used in the main work, but samples from commerce were tested and found to be of similar toxicity. If any solid material were present in the solution it was likely to be very dangerous. Some Congo red dye issued as an indicator was tested and found to have no greater toxicity and a pH nearer to the U.S.P. range than one issued for injection. The indicator dye passed the B.P.C. test for pyrogenicity with ease. Seven months at least were laid down for storage time. Solutions which had been kept since 1953 had not increased in toxicity.

DR. G. F. SOMERS, in reply, said that the material used by the authors had passed the B.P.C. test. On evaporation if a concentration of 1·5 per cent. were exceeded then the Congo red would readily precipitate. If stored in a refrigerator there was a danger of crystallisation of the dye; the crystals tended to grow and were difficult to redissolve.