SOME OBSERVATIONS ON TOXICITY TESTS OF ANTIBIOTICS

By

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Considerable difficulty has been experienced in interpreting the results of toxicity tests on certain antibiotics employing aqueous solutions as recommended by various Pharmacopoeias. The strain of mice as a possible factor influencing the results has been emphasised. Ionic concentration of the solutions and the rate of injection are other factors considered responsible for the immediate mortality in the strain of mice employed in these studies.

Using isotonic solutions prepared in normal saline or glucose solution and injecting 0·5 ml, or making the solutions isotonic by increasing the concentration *per se* of the antibiotic in water and injecting lesser volume than what is recommended, it was possible to obviate the difficulties encountered. Phenobarbitone and mepyramine maleate decreased the mortality rate due to intravenously administered procaine penicillin when tested in the manner described.

Though normal saline is the vehicle of choice for preparing solutions in most pharmacological studies as well as routine tests involving intravenous administration of drugs, there exists some ambiguity regarding the choice of the diluent for toxicity tests of antibiotics as prescribed in different Pharmacopoeias. The British Pharmacopoeia (1953) and the Pharmacopoeia of India (1955), for instance, prescribe water or saline as a diluent for carrying out the "Abnormal toxicity" of antibiotics thus leaving the choice of the diluent to the experimenter. The Pharmacopoeia of United States of America (1960) on the other hand, recommends the usage of normal saline for "Safety test for antibiotics" unless otherwise stated in the individual monograph.

Semenza and Soncin (1957) observed that the LD_{50} of intravenously administered tetracyclines in mice and a few other species of animals shifted considerably depending upon whether saline or distilled water was utilised as the diluent but not of streptomycin. Based on this they had recommended increasing the doses of tetracyclines to be used in the "Safety" tests and to employ normal saline as a solvent instead of water, possibly with the idea of making the toxicity tests for tetracyclines more effective. They suggested that a safety margin existed with the doses laid down by U.S.P. and B.P.

which excluded a possible interference of the potentiating effects of hypotonic solutions on the toxicity as described by them. However, our experience is otherwise.

In our laboratory we had experienced considerable difficulty in the interpretation of the results of toxicity test on most of the antibiotics when their aqueous solutions were employed for the "Safety" tests even when the test doses and other conditions for the tests recommended by different Pharmacopoeias were strictly adhered to. In some cases, aqueous solutions when administered intravenously produced severe convulsions in mice followed by immediate death. No such reactions could be observed when normal saline was employed for preparing the solutions. As far as we are aware of, there is no mention in the literature of the immediate mortality of mice using aqueous solutions at the dosages recommended by Pharmacopoeias, as is observed by us. Therefore, a study of the choice of the diluent for the routine toxicity testing of some antibiotics was thought necessary.

METHODS

Antibiotics and other materials.—Samples of clinical grade antibiotics of different manufacturers were obtained from drug-store counters. Recrystallized procaine penicillin G and sodium penicillin G used in the study were specially prepared in our Antibiotic Plant. Before use, these samples were evaluated for maximum potency and purity by comparing them with reference standards.

All other materials used in animal tests were of injectable grade, certified by manufacturers for parenteral administration. Prior to use, tests were carried out on mice to ascertain whether all such materials were free from acute toxic reactions adjudged as criteria to be employed in further experimentation.

Animals.—Inbred albino mice of Haffkine Institute origin, weighing between 18-20 g and not previously employed for any tests were used for carrying out the toxicity tests.

Injection technique.—All substances were dissolved either in water or normal saline and the resulting solutions were injected intravenously at a uniform rate within five seconds using groups of 5 animals unless otherwise mentioned. The volume per injection was 0·5 ml per mouse, except where a specific statement was made to the contrary.

Response.—Observations were taken immediately after intravenous administration of the drugs. Criteria for the manifestation of acute toxicity of the material under investigation were two fold : (i) convulsions or other aberrant reactions not observed with saline-injected controls and (ii) death immediately after intravenous administration of the test solutions.

RESULTS AND DISCUSSION

In the first series of experiments some of the more common antibiotics were tested on mice in water and saline. The results of such experiments are shown in Table I. The most significant observation in these experiments was that whereas aqueous solutions of various antibiotics manifested toxic

TABLE I

"Acute toxicity" in mice of certain antibiotics and procaine hydrochloride solutions in water, normal saline and 5% glucose solution

Test materials	Test dose per mouse	Response					
		Water		Normal saline		5% Glucose solution	
		Convulsions	Death	Convulsions	Death	Convulsions	Death
Recrystallized Procaine Peniciliin G	1000 units	5*	3*	0*	0*	0	0
Fortified procaine Penicillin B.P.							
Manufacture A	,,	5	2	0	0	0	0
Manufacture B	,,	5	2	0	0	—	—
Manufacture C	,,	5	3	0	0	—	—
Manufacture D	,,	5	3	0	0	0	0
Recrystallized Sodium Penicillin G	2000 units	3	0	0	0	0	0
Streptomycin Sulphate							
Manufacture A	1000 μg base	5	3	0	0	0	0
Manufacture B	,,	5	4	0	0	—	—
Manufacture C	,,	5	2	0	0	—	—
Dihydrostreptomycin Sulphate	,,	5	2	0	0	0	0
Tetracycline hydrochloride	1000 μg	5	1	0	0	0	0
Procaine hydrochloride	0.4 mg	5	4	0	0	0	0

* Average of twenty groups, each group comprising of five mice.

effects on mice to a greater or a lesser extent, those in normal saline were completely devoid of any such reactions. The nature of the reactions using aqueous solutions varied from mild convulsions in some to very severe ones in others with respiratory failure leading to death.

Under the present set of experimental conditions the phenomenon of convulsive reactions seemed to be a generalised property of all antibiotics tested, while the ultimate death depended largely on the nature of the compounds themselves. Procaine, for example, is known for its central excitant effects leading to convulsions or cardiovascular collapse and therefore, it is quite logical to expect procaine penicillin, a simple salt of procaine and penicillin, to rank high for its toxicity among all the antibiotics tested. That these effects were more due to procaine molecule itself was proved by carrying out tests using aqueous solutions of procaine hydrochloride at a concentration of procaine equivalent to that in procaine penicillin (Table I). Exactly similar reactions were observed which were totally blocked in a like manner when normal saline was used instead of water for preparing the solution. Five per cent glucose exerted similar protective action on al antibiotics tested and also on procaine hydrochloride.

For a given dosage of an antibiotic, it is the volume of water used for injection which governs the severity of the reactions in mice. This fact is borne out from experiments where the same amounts of the antibiotics were administered to individual mouse in varying volumes of water (Table II). Procaine penicillin, the most toxic of all antibiotics tested, failed to produce

TABLE II

Effect of the volume of water used for injection on the "Acute toxicity" of certain antibiotics and procaine hydrochloride

Test materials	Test dose per mouse	Response									
		Convulsions					Death				
		0.5 ml	0.4 ml	0.3 ml	0.2 ml	0.1 ml	0.5 ml	0.4 ml	0.3 ml	0.2 ml	0.1 ml
Recrystallized procaine penicillin	1000 units	4	3	3	0	—	3	0	0	0	—
Streptomycin Sulphate	1000 μg base	5	4	2	0	—	2	0	0	0	—
Dihydrostreptomycin Sulphate	1000 μg base	5	2	0	0	—	1	0	0	0	—
Recrystallized Sodium Penicillin G	2000 units	5	2	0	0	—	0	0	0	0	—
Procaine hydrochloride	0.4 mg	5	—	—	2	0	4	—	—	0	0

toxic reactions in any of the mice when injected in an aliquot of 0.2 ml of water. A graded response was observed at intermediate levels between 0·2ml and 0.5 ml, the latter being the recommended volume for toxicity testing. Convulsions and death were totally absent when other antibiotics were similarly tested in 0·2 ml of water. These results show that sodium chloride has no particular influence in reducing the immediate mortality from intravenously administered antibiotic solutions because the same effect can be obtained by increasing the concentration of the antibiotics in the aqueous solution. These findings can be explained to some extent on the basis of ionic concentration of the solutions. This fact was verified by simple calculations in each case by applying the formula M/3.5 n to determine the ionic concentration where 'n' is the number of ions into which the molecule dissociates (Gaddum, 1959). In all the cases where the volume of water was reduced to 0.2 ml the solutions approached isotonic concentrations. In view of these experiments the general recommendations in the Pharmacopoeias of 0.5 ml of water wherever suggested, might not hold good for all compounds and must be based on the molecular weight and ionising property of the particular compound in question. Alternately, the solutions may be prepared in isotonic saline or glucose if it is desired to keep the volume of the injection constant at 0.5 ml per animal. Using solutions which allowed injection of doses near the LD_{50} in a volume of the solution 5 times smaller (0.1 ml instead of 0.5 ml per animal), Semenza and Soncin (1957) found that the mortality due to intravenously administered tetracyclines was constantly less as compared with the same at usual concentrations. This lends support to our observations which are likely to have an important practical significance in the routine toxicity testing of antibiotics.

Further experiments carried out with different antibiotics revealed the fact that the severity of the toxic reactions could be greatly reduced by increasing the recommended time of injection from "within 5 seconds" to about 30 seconds (Table III). The rate of intravenous injection is known to alter profoundly the toxic effects of certain drugs. Thus the fatal dose of sodium amytal declined sharply with increased speed of injection and the safety margin varied widely in mice depending on the rate of injection used (Swanson and Shonle, 1931). A very rapid injection of a few ml of an animal's own blood has been reported to produce wide spread disturbances, named "speed shock" with a possible early death (Hirschfelder *et al.*, 1931). The results on toxicity tests using aqueous solutions are in complete agreement with this fact on the rate of injection. However, for a given rate of injection, solutions in normal saline are better tolerated than aqueous solutions.

TABLE III

Effect of the rate of injection on "Acute toxicity." Injection time 30 seconds

Antibiotic	Test dose per mouse	Response	
		Convulsions	Death
Recrystallized Procaine Penicillin G	1000 units	2	0
Streptomycin Sulphate	1000 μg base	3	0
Dihydrostreptomycin Sulphate	1000 μg base	2	0
Recrystallized Sodium penicillin G	2000 units	1	0

Table IV shows the effect of mepyramine maleate, prednisolone and phenobarbitone in decreasing toxic effect of procaine penicillin. Phenobarbitone appears to be better than others followed by mepyramine maleate. A combination of these two drugs, however, failed to yield any better results indicating thereby that their effects were not additive. It is pertinent to mention in this connection the work of Rothschild and Rochae Silva (1954) on anaphylatoxin. Simply by increasing the sodium chloride concentration of plasma to twice the ionic strength of normal saline they were able to completely block the activation of anaphylatoxin in plasma by agar. As opposed to this they were able to bring about a spontaneous activation of anaphylatoxin by simple dilution of the plasma with distilled water or reduction of its ionic strength by dialysis against distilled water. This being the case *in vitro*, the possibility of similar mechanisms operating *in vivo*, as in the case of the present studies, cannot be ruled out. The aspects on the elucidation of the mechanism of action of the drugs and the search for controlling agents if any, has not been the sole purpose of our studies and needless to say, further experiments are necessary to throw light on these and possibly other interesting phenomena.

The findings reported here seem to be contradictory to some extent to what is suggested in the Pharmacopoeias. The recommendation of normal saline for the "Safety test" of procaine penicillin by U.S.P. is in general agreement with our results. But aqueous solutions suggested in I.P. and U.S.P. for streptomycin and sodium penicillin produced convulsions in general and death in particular cases immediately after the administration. No such aberrant reactions were observed when normal saline was employed instead. 0.5 ml of water alone when administered intravenously failed to

TABLE IV

Effects of mepyramine maleate, prednisolone and phenobarbitone on the toxicity of aqueous solution of recrystallized procaine penicillin G (1000 units/mouse)

Drugs	Concentration	Method of administration	Experimental		Control		Difference	
			Convulsions	Deaths	Convulsions	Deaths	Convulsions	Deaths
Mepyramine maleate	(i) 25 μg	Together with procaine penicillin intravenously.	3	3	4	2	−1	+1
	(ii) 100 μg	Intraperitonial, 15 min. prior to procaine penicillin injection.	5	4			+1	0
	(iii) 200 μg	,,	3	2	4	4	−1	−2
	(iv) 400 μg	,,	3	2			−1	−2
Prednisolone	25 μg	Together with procaine penicillin intravenously.	2	1	4	2	−2	−1
Phenobarbitone	(i) 0.5 mg	Intraperitonial, 30 min. prior to procaine penicillin injection.	2	1			−2	−3
	(ii) 1.0 mg	,,	4	2	4	4	0	−2
	(iii) 1.5 mg	,,	5	1			+1	−3
Mepyramine maleate + Prednisolone	25 μg + 25 μg	Together with procaine penicillin intravenously.	3	3	4	4	−1	−1
Phenobarbitone + Mepyramine maleate	(i) 1.0 mg + 200 μg	Intraperitonial, 30 min. prior to the test) Intraperitonial, 15 min. prior to the test)	5	4			0	0
	(ii) 1.0 mg + 400 μg	,,	5	2			0	−2
	(iii) 1.5 mg + 200 μg	,,	5	1	5	4	0	−3
	(iv) 1.5 mg + 400 μg	,,	3	2			−2	−2

produce any comparable toxic effects. Therefore, water by itself as a possible cause of the effects is ruled out. The only plausible explanation is the strain to strain variation in mice In our experiments occasionally some mice did not manifest any reactions and finally survived the test while certain others, apparently not showing any reactions, finally succumbed to the drugs. These might be extreme cases of insensitivity and hypersensitivity of individuals within the same population. This being the extent of biological variation within the same strain of mice, it is quite likely that certain strains used in other laboratories are altogether different in their behaviour to these and possibly other drugs. That the strain of mice affects the results when testing antibiotics for freedom from undue toxicity had been clearly brought out by the work of Nir—Grosfeld *et al* (1958). Even though there is no mention of immediate mortality in mice as is implied in the present studies their results are clearly indicative of the paramount importance of the strain for the toxicity tests. The fact that atleast there are some strains which are extremely sensitive to antibiotics is borne out from our studies In view of these and other studies referred to, the recommendations made in various Pharmacopoeias are likely to prove restrictive in the interpretation of the results of toxicity tests. In addition to the description of the conduct and criteria for toxicity testing, the present studies suggest that the specific strain to be used must also be indicated to avoid any conflicting results between different laboratories.

The suitability of normal saline in preference to water as a diluent for other drugs for which toxicity testing is a Pharmacopoeial obligation needs to be varified before any generalisation could be made. However, our studies are indicative of the fact that normal saline may obviate all possible discrepancies and is therefore recommended for the toxicity testing of antibiotics.

The results reported here seem to have some clinical importance. Sudden death immediately after injection of certain antibiotics, particularly procaine penicillin are not uncommon. Bell (1954) reported a case of sudden death following intramuscular injection of a single dose of procaine penicillin, presumably due to accidental administration of the drug into the blood vessel. In view of the findings reported here, it is felt that it may be safer to inject the antibiotics using saline rather than water.

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ANNOUNCEMENT

As a result of the situation arising due to the outbreak of hostilities on our northern borders it was decided, with the greatest reluctance, to cancel the 8th Annual Conference of the Association of Physiologists and Pharmacologists of India. The following decisions have been taken by correspondence :—

1. The 9th Annual Conference of the Association will be held at Jaipur in December, 1963—January, 1964.
2. The office bearers elected for 1962 will continue to hold their respective offices in 1963.
3. The audited accounts of 1962 will be approved by the Executive Committee and will be placed in the 9th. Annual General meeting of the Association.
4. The auditors appointed for 1962 will continue for 1963.
5. The abstracts of the papers will be published in the Journal of the Association.
6. The new members elected during 1962 were approved.

The inconvenience caused to the members and the various participants is very much regretted.

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