SALINE SOLUTION AS A FACTOR AFFECTING THE TOXICITY OF INTRAVENOUSLY INJECTED TETRACYCLINES IN MICE

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THE toxicity of tetracyclines is generally tested by intravenously injecting five mice of uniform weight with 0·5 ml. of a solution containing 2 mg./ml. of the antibiotic under test, in the form of the hydrochloride, over a period of not more than five seconds.

The regulations laid down by the Food and Drug Administration[1] and almost universally accepted, state that the solution of the antibiotics (chlortetracycline and tetracycline) must be prepared in sterile, distilled water, and the mortality must be observed during the 48 hours following injection. These regulations are common to the toxicity tests for other antibiotics: penicillin (alone or combined with vasoconstrictors), streptomycin (alone or combined with penicillin) and dihydrostreptomycin. The use of sterile physiological salt solution is instead prescribed for procaine penicillin, dibenzylethylenediamine-penicillin, penicillin V, chloramphenicol and bacitracin.

The United States Pharmacopeia, XV, does not greatly change the Food and Drug Administration regulations as far as the safety tests for antibiotics are concerned. A solution of sodium aminoacetate must be used for oxytetracycline.

In contrast, the British Pharmacopoeia, 1953, in the test for undue toxicity of chlortetracycline, directs that the solution of the antibiotic may be made either in water or in saline solution. However, it does not specify the rate of injection. In the 1955 Addendum these regulations are not modified.

We would like to draw attention to the importance of using either one or the other solvent in determining the acute toxicity of tetracyclines in mice, by intravenous administration. In fact, we have observed large differences in the mortality rate of animals injected intravenously with tetracyclines, whether distilled water or physiological solution is used as solvent. No record has been found in the literature but Dr. Grove told one of us that he obtained similar results with chlortetracycline.

MATERIALS AND METHOD

CF–1 mice and CF Wistar rats were used for the experiments. Both were bred in our laboratories.

Unless otherwise described, each mouse was injected with 0·5 ml. of solution per 20 g. body weight, the concentration of the drugs being suitably varied, in order to inject the same volume of liquid for each dose. The rate of injection was always 0·1 ml./sec. All injections were by one experimenter and the time was controlled with the aid of a metronome.

Four hours before the test, food was withdrawn and, after injection, the animals were placed, in groups of five, in cages kept in well-ventilated surroundings at a temperature of 23° (±2°). Observations were made for 72 hours. Toxicity tests on the same substance, using different solvents, were carried out on the same day or, at the latest, after 24 hours. Oxytetracycline, chlortetracycline, tetracycline and bromtetracycline were used in the form of the hydrochlorides, and streptomycin as the sulphate. The distilled water and the saline solutions were sterile and pyrogen-free.

The LD50 and confidence limits 19/20 were calculated according to Lichtfield and Wilcoxon[2].

The technique for the *in vitro* and *in vivo* haemolysis tests are described later on.

Results

The intravenous LD50 of tetracycline, oxytetracycline, chlortetracycline, bromtetracycline and streptomycin was determined in mice, using both water and physiological salt solution. As seen in Table I, the values of the LD50 differ significantly when a tetracycline is administered in aqueous instead of saline solution.

TABLE I

LD50 OF THE FOUR TETRACYCLINE HYDROCHLORIDES AND OF STREPTOMYCIN SULPHATE, INTRAVENOUSLY INJECTED TO MICE EITHER IN AQUEOUS OR IN SALINE SOLUTION

Drug	Solvent	Doses	Animals/ dose	LD50 mg./kg.	C.L.*	T.R.†	C.L.‡	Significance of difference (P = 0·05)
Tetracycline HCl (sample 57)	Water	4	20	149	137–162	1·31	1·21–1·41	+
Tetracycline HCl	NaCl 9 g/l.	4	20	195	186–204			
Tetracycline HCl (standard)	Water	4	25	157	144–172	1·42	1·30–1·55	+
Tetracycline HCl	NaCl 9 g/l.	4	25	223	211–236			
Oxytetracycline HCl	Water	6	20	124	114–134	1·43	1·32–1·54	+
Oxytetracycline HCl	NaCl 9 g/l.	4	20	177	169–185			
Chlortetracycline HCl	Water	4	20	101	94–110	1·21	1·07–1·32	+
Chlortetracycline HCl	NaCl 9 g/l.	4	20	122	113–132			
Bromtetracycline HCl	Water	4	20	83	72–95	1·44	1·20–1·72	+
Bromtetracycline HCl	NaCl 9 g/l.	4	20	120	106–136			
Streptomycin H_2SO_4	Water	3	20	120	107–134	1·13	0·98–1·30	–
Streptomycin H_2SO_4	NaCl 9 g/l.	4	20	106	96–117			

* C.L. = Confidence Limits (P = 0·05) of LD50. † T.R. = Toxicity ratio.
‡ C.L. = Confidence Limits (P = 0·05) of the T.R.

The value of LD50 of the same sample of tetracycline dissolved in different concentrations of NaCl (1, 3, 5, 7, and 9 g./l.) again showed wide differences (Table II, Fig. 1). These results strongly suggest a

relation between the concentrations of NaCl in the solvent and the values of the LD50.

The difference in the acute toxicity of tetracyclines in mice was also observed for other species of animals. On intravenously injecting into rats equal volumes of tetracycline solution, corresponding to 2 ml./200 g. of body weight, we obtained a significant difference of the LD50, according to the solvent used. These differences tend to decrease when the volume injected is reduced. As can be seen from Table III, using a volume of 1 ml./200 g. of body weight, the differences are no longer significant.

TABLE II

LD50 OF TETRACYCLINE HCl IN SALINE SOLUTIONS AT VARYING CONCENTRATIONS

Solvent g./l.	LD50 mg./kg. of tetracycline and Confidence Limits
Water	112 [96–130]
NaCl 1	142 [133–152]
,, 3	155 [138–174]
,, 5	172 [155–191]
,, 7	195 [164–232]
,, 9	210 [189–233]

Two groups of five guinea pigs—male and of the same weight (450 g.)—were used to determine the lethal dose of tetracycline, by perfusion into the jugular vein. A constant rate perfusion apparatus was used (3·06 ml./minute). The time of death was checked by electrocardiographic recording. The rate of perfusion and the concentration of the tetracycline solutions were chosen in such a way that the deaths occurred after a reasonably long interval.

TABLE III

DIFFERENT LD50 VALUES OF TETRACYCLINE HYDROCHLORIDE INTRAVENOUSLY ADMINISTERED INTO RATS, ACCORDING TO THE VOLUME INJECTED

Substance	Solvent	ml./200 g. rat	Doses	Animals/ dose	LD50 mg./kg.	C.L.*	T.R.†	C.L.‡	Significance of difference (P = 0·05)
Tetracycline HCl (sample 57)	Water	1	4	10	130	84–202	1·14	0·74–1·77	–
Tetracycline HCl	NaCl 9 g./l.	1	4	10	149	139–160			
Tetracycline HCl (sample 57)	Water	2	4	10	120	107–134	1·46	1·27–1·68	+
Tetracycline HCl	NaCl 9 g./l.	2	4	10	175	160–191			

* C.L. = Confidence Limits (P = 0·05) of LD50. † T.R. = Toxicity ratio.
‡ C.L. = Confidence Limits (P = 0·05) of the T.R.

The results of these experiments (Table IV) confirmed that even in both these species of experimental animals, and under different experimental conditions, the LD50 showed significant changes according to the selected solvent.

TABLE IV

MEDIUM LETHAL DOSES OF TETRACYCLINE HCl BY PERFUSION INTO THE JUGULAR VEIN OF GUINEA PIGS

Solvent	Concentration and pH	Infusion rate	Animals	Median lethal doses mg./kg. ± S.D.
Water	3·5 g./l.–3·2	3·06 ml./min.	5	259 ± 24·8
NaCl 9 g./l.	3·5 g./l.–3·2	3·06 ml./min.	5	449 ± 38·5

Further proof of the importance of the hypotonicity of solutions in affecting tetracycline LD50 values, was obtained by administering to groups of mice, tetracycline dissolved in water, at concentrations higher than in above-mentioned tests.

Using solutions which allowed injection of doses near the LD50 in a volume of solution five times smaller (0·1 ml., instead of 0·5 ml./animal), we found that there was a marked difference in the mortality of the animals treated (Table V). The solubility of tetracycline did not allow the determination of the LD50 using high concentrations, but the differences in mortality for the single doses were undoubtedly significant. In fact, using concentrated solutions, mortality was constantly less than that resulting from corresponding doses at the usual concentration. This fact was not observed when tetracycline dissolved in physiological salt solution was used.

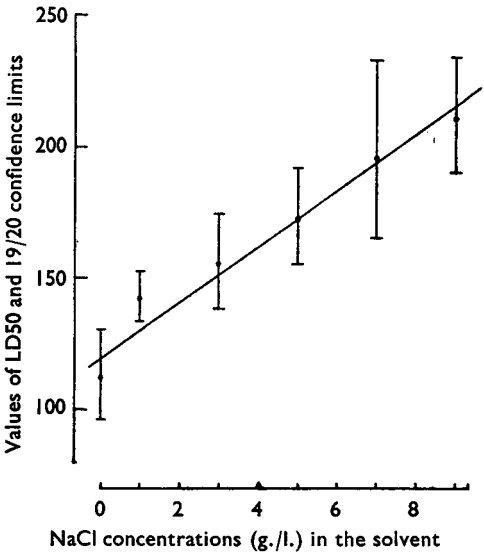

FIG. 1. LD50 values for mice given tetracycline-hydrochloride i.v. in saline solutions of different concentration.

These results show, as could logically be expected, that NaCl has no influence in reducing the immediate mortality from intravenously injected tetracyclines, as the same effect can be obtained by increasing the concentration of the antibiotics in the solutions. On the other hand, it appears that the volume of solution injected has some influence, independent of the molecular concentration.

On the assumption that distilled water may influence the pattern of toxicity produced by intravenously injected tetracyclines, the mechanism of the toxic action could be logically explained by the modifications that hypotonic solutions may produce in the homeostasis of the circulating blood. This effect would be always noticeable when water is used as a solvent for substances which cause alterations of the haematic constants.

We therefore studied the acute toxicity of saponin which has a well-known haemolytic action, and of hydrochloric acid solution, under experimental conditions analogous to those described above. The acute toxicity of these substances was greater when they were administered in aqueous solution. The LD50 values for saponin, intravenously injected into mice, when calculated on the basis of the mortality observed during the 72 hours after treatment, are not significantly different for the two solvents used. If, however, the immediate mortality is considered (within

TABLE V

VARIATIONS OF THE MORTALITY BY TETRACYCLINE HCl ACCORDING TO THE CONCENTRATION OF THE SOLUTIONS INTRAVENOUSLY INJECTED INTO MICE

Drug	Dose mg./kg.	Solvent	Volume injected: 0·5 ml./mouse					Volume injected: 0·1 ml./mouse				
			Per cent concentration	pH	M/1	Treated animals dead	Per cent mortality	Per cent concentration	pH	M/1	Treated animals dead	Per cent mortality
Tetracycline HCl	110	Water	0·44	3·3	0·009	13/20	65	2·2	2·9	0·046	7/20	35
Tetracycline HCl	130	Water	0·52	2·8	0·011	5/10	50	2·6	2·2	0·054	2/10	20
Tetracycline HCl (sample 57)	140	Water	0·56	3·2	0·012	7/20	35	2·8	2·6	0·058	0/20	0
Tetracycline HCl	180	Water	0·72	3·1	0·015	15/20	75					
Tetracycline HCl	250	Water						4·0	2·4	0·083	8/20	40
Tetracycline HCl	110	NaCl 9 g./l.	0·44	3·3	0·162	1/20	5	2·2	2·9	0·199	5/20	25
Tetracycline HCl	130	NaCl 9 g./l.	0·52	2·8	0·164	1/10	10	2·6	2·2	0·207	3/10	30

two hours from injection), it is seen that there are no deaths among the animals treated with saponin in a salt vehicle, while in those treated with aqueous solution mortality was sufficiently high to allow determination of the LD50 (Table VI).

We did not study this interesting experimental fact further, as we felt that it was already possible to establish an analogy between the phenomenon described for the tetracyclines and the results of the immediate

TABLE VI

EFFECTS OF TIME ON THE TOXICITY OF SAPONIN* DISSOLVED IN DISTILLED WATER AND IN NORMAL SALINE

Drug	Solvent	Doses	Animals/ dose	LD50 mg./kg. and Confidence Limits	
				2 hours	72 hours
Saponinum	Water	7	20	27 (18–41)	8·6 (7·2–10·2)
Saponinum	Normal saline	5	25	>50	8·0 (6·1–9·6)

* Saponinum–Merck.

toxicity from saponin, particularly in view of the fact that toxic doses of tetracyclines cause 70 per cent of the deaths within the first 20 minutes after injection.

We then injected intravenously, as above, a toxic dose of hydrochloric acid in distilled water (0·5 ml. of N/4 solution) to two groups of mice weighing 20 g. each. The same dose was repeated, using saline solution and checking potentiometrically the pH which had to be identical in both cases. We found that 13 out of 20 mice (65 per cent) died after 72 hours, when we used aqueous acid solution, while only three out of 20 (15 per cent) died when the same solution contained NaCl.

We feel that these results are sufficiently significant to allow the conclusion that, even in the case of hydrochloric acid solution, the presence of distilled water, i.e., the hypotonicity of the solution, unfavourably influences the resistances of mice to intravenous injection of acid solutions.

As the injection of distilled water alone in volumes equal to those used for tetracycline, did not cause any evident disturbances in mice, the mechanism of action by which distilled water increased in toxicity of these substances was still to be ascertained.

In vitro blood red cell resistance tests were made with rabbit red cells washed and suspended in different concentrations of NaCl, ranging from 0·9 to 0·3 per cent. For adjustment and maintenance of a neutral pH, a phosphate buffer was used. The effective molecular concentration was calculated after the buffer had been added.

No distinct change in the cell resistance in the presence of tetracycline was observed up to concentrations of 416 μg./ml. However, we observed that when control of the red cells suspensions in progressively increasing hypotonic solutions was continued for 16 and 24 hours, at room temperature, there was a decrease of globular resistance in the tubes containing tetracyclines, whilst the values of the maximum haemolytic concentration remained almost constant in the controls.

This point is of limited importance, as in these studies interest was centered on the immediate effect of the tetracyclines. However, it might perhaps indicate a tendency to affect the haematic crasis.

We also examined the behaviour of mice red cells in hypotonic solutions *in vitro*, after treating the animals with tetracycline, but found no difference in the globular resistance compared with the untreated controls.

However, during these tests, we observed that, independently of the globular resistance of the whole cells, the serum of animals treated with toxic doses of tetracycline showed a certain degree of directly observable spontaneous haemolysis. In order to confirm this, we injected toxic doses of tetracycline in physiological saline solution and in distilled water into groups of mice. The tetracycline dose was 190 mg./kg., and the concentration and volume of the solution injected were those normally used in these experiments.

The animals were killed exactly one minute after the end of the injection. After rapid opening of the abdomen and evisceration, 0·1 ml. of blood was drawn out of the abdominal aorta, immediately diluted with 3 ml. of physiological solution and centrifuged for 10 minutes at 1000 r.p.m. After centrifuging it was observed whether the supernatant fluid contained dissolved haemoglobin.

We noticed that in all of the 11 animals treated with tetracycline dissolved in water, the blood showed intense haemolysis, whilst this was seen only in three out of 11 animals treated with tetracycline dissolved in physiological salt solution. We considered the direct observation sufficient, without further determining quantitatively the haemoglobin present.

These tests, although rough and ready, indicate that the presence of distilled water is not without importance, having an influence on the directly observable phenomenon of the lysis of mouse red cells. We do not feel that the phenomenon of haemolysis and that of death must be, in this case, directly connected. In normal conditions, i.e., when

distilled water alone is intravenously injected, there is also a haemolytic effect without death of the animal. However, this tangible aspect of the action of hypotonic solutions may play a part as an accessory factor in causing death in acute intravenous toxicity tests.

Discussion

It is logical to believe that the intravenous administration in hypotonic solution of a given substance, by itself capable of altering the haematic homeostasis, increases the toxic effect and the death rate of the treated animals.

Distilled water alone is not capable of causing death at the doses considered (0·5 ml. per 20 g. of mouse). It also does not affect the mortality produced by toxic doses of drugs which (owing to their molecular weight and the doses at which they may be usefully employed in toxicity tests) do not cause hypotonic conditions or (owing to their pharmacological properties) have no tendency to alter the haematic balance. The rational interpretation of the experimental results suggests some practical conclusions. It is not justifiable to deduce that a given substance whose mechanism of immediate toxicity is unknown, must show, when rapidly injected intravenously into a small animal using a considerable volume of solvent, the same toxicity in water or saline solution. Although it is not excluded that the two data may sometimes be identical, this possibility must still be experimentally tested.

For practical purposes, it is thus essential to specify the solvent to be used in the various "safety" and "toxicity" tests of different official publications, as it is not logical to leave the choice of distilled water or saline solution to the experimenter.

As regards the control tests on tetracyclines, our results have led us to believe that, by using aqueous solutions, the toxic phenomena possibly occurring are not completely attributable to these drugs. In fact it has to be considered that, also at different and lower doses, there is reproduction of those experimental conditions which we have shown to be the least suitable for determining the toxicity of tetracyclines.

In our opinion, it would be more correct to increase the doses of tetracyclines to be used in the "safety" tests, and to employ as a solvent NaCl solution instead of distilled water, although the safety-margin existing with the doses laid down by the U.S.P. and the B.P. exclude a possible interference of the potentiating effects of hypotonic solutions on the toxicity, as described by us.

Summary

1. Tetracycline, chlortetracycline, oxytetracycline and bromtetracycline all show, on intravenous administration to mice, less toxicity when dissolved in saline solution than in distilled water.

2. LD50 values have been determined using both water and saline solution to dissolve the antibiotics. They differ significantly according to the solvent used.

3. Analogous results have been obtained with different species of animals. It is concluded that this phenomenon is directly dependent on the experimental conditions used in performing toxicity tests of tetracyclines and other antibiotic substances.

REFERENCES

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