BRITISH PHARMACEUTICAL CONFERENCE OXFORD, 1954

SCIENCE PAPERS AND DISCUSSIONS

(*continued from page 905*)

A METHOD FOR STUDYING PERCUTANEOUS ABSORPTION IN THE RAT

BY J. W. HADGRAFT and G. F. SOMERS

From the Royal Free Hospital and the School of Pharmacy, University of London

Received July 12, 1954

WHILE considerable progress has been made in the discovery and formulation of new ointment bases, much has still to be learnt of their effects on the skin. An ointment consists of two distinct parts—the base itself and the incorporated medicament. Absorption of the medicament through the skin may or may not be desirable, and it will be influenced by the nature of the base and by the chemical and physical properties of the drug itself.

Many different methods have been described for studying the absorption of drugs applied to the intact skin, but most of them are open to criticism. Their usefulness compared with clinical studies are also doubtful, but they provide valuable information in comparing the relative effectiveness of different ointment bases. *In vitro*[1] methods can be dismissed as being quite useless in providing reliable information on skin absorption. Early methods for studying skin absorption were based on the urinary excretion of some easily recognised substance such as potassium iodide or a salicylate.[2,3,4,5] They cannot be very sensitive and the results are influenced by many extraneous factors. The introduction of radioactive tracer substances, which can be readily detected even in minute quantities, provides a new line of approach with great potentialities.[3] Unfortunately few people have the special equipment and facilities for handling radioactive material. Histological studies[6,7] have given important information on skin penetration, but they do not give a measure of systemic absorption. They have shown the distribution of the materials within the skin structures, and have indicated that the most important route of absorption is through the sebaceous glands.

Pharmacologically active drugs, such as morphine and strychnine, have been applied to the skin of mice and their physiological effects observed.[8] We have devised such a method, based on the action of eserine in depressing the activity of the enzyme cholinesterase in the rat. The rat when injected with acetylcholine secretes opaque reddish brown tears. This phenomenon of chromodacryorrhœa was first described by Freud,[9] and

it was shown that the sensitivity of the rat is increased considerably by eserine.[10] The response has been used for an accurate quantitative assay of anticholinesterase drugs.[11] We have found it to be a convenient method for measuring percutaneous absorption and we are using it to study the absorption of eserine and its salts from various ointment bases. This paper describes the method and some of the results which we have obtained.

Materials

Male albino rats weighing 100 to 200 g. were used, since these have been reported to give the most satisfactory and consistent results. The standard acetylcholine solution was prepared by dissolving 100 mg. of acetylcholine chloride and 100 mg. of $KH_2PO_4,12H_2O$ in 100 ml. of a 0·8 g./100 ml. solution of sodium chloride. This solution has *p*H 4·1 to 4·3 and is stable for several weeks if kept in the refrigerator. Fresh dilutions of this standard solution were prepared in 0·9 per cent. sodium chloride solution each time the injections were made.

Method

The hair was removed over an area of approximately 25 sq. cm. of the skin of the back of the rat, using clippers to avoid trauma of the skin which is common after shaving or using depilatory pastes. The rats were placed into separate tins to prevent contamination by contact with each other. The initial sensitivity of the rats was determined by injecting subcutaneously a dose of 50 μg./100 g. of acetylcholine solution and any rats which produced red tears were regarded as too sensitive and not used in the experiment. A weighed amount of the ointment base (250 mg.), containing a known concentration of eserine, was applied to the whole area of denuded skin and rubbed well in for 30 seconds using the finger enclosed in a rubber finger stall. The rats were then kept under constant observation to prevent them licking their backs. In most experiments 24 rats were used, 3 groups of 4 for the test preparation and 3 groups of 4 for the standard. 3 concentrations of eserine were applied in the ointments, in the same ratio 0·5, 0·25 and 0·125 per cent. Eserine base dissolved in white soft paraffin was used as the standard for comparison in most of the experiments which we have so far carried out on the oily bases. The response to 50 μg. of acetylcholine was measured again 30, 60 and 120 minutes after the inunction. The red tear response was measured by inserting a small piece of filter paper into the corner of each eye and it was convenient to stick these pieces on to a sheet of graph paper before assessing the response obtained. The magnitude of the response was scored as follows[11]:— a very red and copious secretion, 4; a red but not copious secretion, 3; pink or a trace of pink, 2; a slight trace of pink, 1.

Experimental

Sensitivity to acetylcholine. Suitable doses of acetylcholine for the experiments were first determined. Burgen[11] reported 300 μg./100 g. of bodyweight to give a red tear response in normal rats and after eserine

the threshold fell to 35 μg. of acetylcholine or less. We found a dose of 50 μg. of acetylcholine to be most suitable for our experiments. This dose did not give a response in normal rats, except when they were unduly sensitive. If a red tear response was obtained at this dose level in the preliminary test, the rat was not used in the experiment. An occasional rat did not give a red tear response even with large doses of acetylcholine, so all new rats were first tested at a dose level of 300 μg./100 g. and discarded as being insensitive if no response was obtained.

Contamination effects. Absorption of eserine can occur by the oral route due to the rats licking their backs. This could only be prevented by keeping the rats under constant observation during the test. Covering the skin proved impracticable with large numbers of rats, and there was the objection that a rise in skin temperature would occur which would increase the rate of absorption. No really satisfactory way of restraining the rats has been found, and anæsthesia affected the results.

Frequency of injections. Acetylcholine should not be given more frequently than once in 30 minutes. Injections at 30, 60 and 120 minutes after applying the ointment were found to be most satisfactory.

Repeating the experiments. The inhibition of cholinesterase by eserine is reversible and the rats could be used again with weekly intervals between the tests.

Evaluation of the response. The lowering of the blood cholinesterase will be determined by 3 main factors—the concentration of eserine in the ointment base; the time over which the observations are made and the rate of absorption through the skin. A maximal effect is often obtained in 30 minutes with the highest concentration of eserine, but not with the lower concentrations. The responses for each rat were therefore summated over the 2-hour period and these totals used in the final calculations.

Calculation of the results. It was not expected that accurate quantitative assays could be made of all ointment bases. Different bases influence the rate and magnitude of absorption of eserine and the standard and test have not the same constitution. These differences confound the basic principles of biological assay[12] which state that the active principle must be the same in the standard and test and that neither should contain any substance which in any way modifies the behaviour of the active principle. However, some form of statistical evaluation of the results obtained was desirable, and the experiments were designed to enable this to be done.

Table I shows the results obtained in an experiment comparing arachis oil (test) with white soft paraffin (standard). These results have been analysed (Table II) by the usual procedure for a 6-point assay,[13] and the sums of squares between doses subdivided by the use of polynomial coefficients. We may conclude that:—(*a*) there is a significant regression between the red tear response and the log. concentration of eserine in the ointment; (*b*) there is no significant deviation from parallelism between the two separate response lines; and (*c*) the curvature of the combined regression line is not significant and the difference in curvature of the two response lines is not significant.

TABLE I

TOTAL RED TEAR RESPONSE OF RATS

Base	Standard			Test		
	White soft paraffin			Arachis oil		
Concentration of eserine (per cent.)	0·125	0·25	0·50	0·125	0·25	0·5
Response (total red tear secretion per rat)	5	6	8	8	12	10
	10	9	12	4	4	10
	1	6	4	4	4	12
	1	5	8	7	5	12
Dose totals	17	26	32	23	25	44

TABLE II

ANALYSIS OF VARIANCE OF THE DATA OF TABLE I

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
Between doses	5	107·7	—		
Difference between preparations	1	12	12	1·37	>0·2
Linear regression	1	81	81	9·27	<0·01
Departure from parallelism	1	2·25	2·25	0·26	>0·2
Curvature of combined curve	1	4·08	4·08	0·47	>0·2
Differences of curvatures	1	8·33	8·33	0·95	>0·2
Within doses (error)	18	157·3	8·74	—	—
Total	23	265			

The test therefore provides a valid assay and calculation of the relative potency of the two preparations gave a ratio of 1 to 1·5. The value of s —the standard deviation of a single observation—was 2·95 and the value for b—the slope of the regression line—was 30. The value for s/b or λ, which is the index of precision, was 0·1, which is high. Calculation gave fiducial limits of error ($P = 0·05$) from 45 to 341 per cent. Thus a large number of animals would have to be used to obtain a high degree of precision. Repeating this experiment gave a ratio of standard to test of 1·45 which is in very good agreement with the first result. These experiments suggest that the absorption of eserine base is better from arachis oil than from white soft paraffin, a result which might be expected.

Unfortunately not all experiments have been as successful as these. One particular difficulty has been the occurrence of one or more rats in a group which did not give a red tear response after eserine. This occurred most frequently in the groups receiving the lowest concentration of eserine. One solution is to convert the results to quantal responses and to use the probit transformation, but this was not satisfactory due to a marked deviation from parallelism between the response lines. If the O responses occurred only in the lowest concentration groups these groups could be omitted and the calculations made for a 2 plus 2 dose assay. Another difficulty which sometimes occurred was a significant deviation from parallelism between the standard and test response lines. It occurred most frequently when the standard and test bases were very different from each other. Such tests did not give valid quantitative estimates and no quantitative conclusions could be made.

Results with other bases. Comparisons have been also made of the absorption of eserine base from solutions in white soft paraffin and in lard. Absorption was better from lard, the ratio being 1·5 to 1·0. A direct comparison of the absorption of eserine base from arachis oil and from castor oil showed better absorption to occur from castor oil, the ratio being 1·7 to 1. Investigations on the absorption of eserine and its salts from other ointment bases are in progress.

Conclusions

Experiments we have carried out so far have shown absorption of eserine base to be better from lard, arachis oil and castor oil than from white soft paraffin. Absorption was better from castor oil than from arachis oil.

Summary

1. A method is described for comparing the absorption of eserine from ointment bases.

2. It is based on the potentiating action of eserine on the red tear response of the rat to acetylcholine.

3. Absorption of eserine base was better from lard, arachis oil and castor oil than from white soft paraffin.

References

1. Reddish, *J. Lab. clin. Med.*, 1929, **14**, 649.
2. Bartenbach, *Berlin Tier Wchsr.*, through *Vet. Rec.*, 1915, 26, July 17th, and *Pharm. J.*, 1915, **95**, 107.
3. Johnston and Lee, *J. Amer. pharm. Ass., Sci. Ed.*, 1943, **32**, 278.
4. Bhatia and Zopf, *ibid.*, 1952, **41**, 542.
5. Bliss, *ibid.*, 1936, **25**, 694.
6. Strakosch, *J. Pharmacol.*, 1943, **78**, 65.
7. MacKee, Sulzberger, Hermann and Baer, *J. Invest. Dermat.*, 1945, **6**, 43.
8. Takashi Seki, *Japan. J. Pharm. Chem.*, 1951, **23**, 138, through *Chem. Abstrs.*, 1951, **45**, 7298b.
9. Freud, *Acta brev. neerl. Physiol.*, 1933, **3**, 159.
10. Tashiro, Smith, Badger and Kezur, *Proc. Soc. exp. Biol., N.Y.*, 1940, **44**, 658.
11. Burgen, *Brit. J. Pharmacol.*, 1949, **4**, 185.
12. Dale, *Analyst*, 1939, **64**, 554.
13. Bliss and Marks, *Quart. J. Pharm. Pharmacol.*, 1939, **12**, 82 and 182.

DISCUSSION

The paper was presented by Dr. G. F. Somers.

The Chairman said that Dr. Somers's method was a promising one and would no doubt be applied to the newer ointment bases.

Dr. K. Bullock (Manchester) suggested the alternative method by the manometric Warburg estimation of blood cholinesterases. Also inhibition by eserine was reversible and eserine was slowly destroyed by the cholinesterase. Would it not have been better to have used one of the irreversible anticholinesterases such as the double quaternary compounds or some of the phosphorus compounds, although the latter were difficult to handle?

MR. D. N. GORE (Dorking) suggested the alternative approach of the use of labelled isotopes.

DR. J. G. DARE (Leeds) said that were Dr. Somers to look on his experiment as quantitative pharmacology as distinct from a biological assay his method was quite valid and beyond reproach.

MR. K. L. SMITH (Nottingham) said that he always used the figure b^2/s^2 as the index of precision. The author said that the value s/b was 0·1 "which was high"; did he mean that 0·1 was high or that the precision of the experiment was high? A figure of 0·1 was quite good for a biological assay and for an accuracy of plus or minus 10 per cent. only 100 animals would be needed. He thought that the experiment could properly be considered a valid biological assay as the authors were trying to see whether eserine was absorbed better from one ointment base than from another and theirs was the only method.

DR. SOMERS, in reply, said they intended to use the method for evaluation of the newer ointment bases but they had tried it out first on well-established bases. It would be applied not only to oily bases but also to emulsified bases containing water. Replying to Dr. Bullock, he said that Warburg manometers were expensive and there would be experimental difficulties with the method. Eserine had been used because its action was reversible and the rats could therefore be used again as, after about a fortnight, they redeveloped their cholinesterases. If irreversible compounds, such as "parathion," were used, the rats could not be used again. Radioactive methods offered a promising field, but many people were unable to acquire the special apparatus needed. Radioactive tracers could be used in animals and also clinically. Replying to Mr. Smith, he said that he had always used s/b, which he regarded as the established method. It would not be practicable to use 100 rats in one experiment.