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A sequential screening test based on the running component of audiogenic seizures in mice, including reference compound PD50 values

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1. The running component of audiogenic seizures in mice has been used as the basis of a sequential screening test for the detection of a variety of centrally acting drugs.

2. For acceptance by the test, an active compound must completely suppress the running component in a total of sixteen mice at a dose of 1/5 LD50 intraperitoneally.

3. Considerable economies in the numbers of animals required for screening have been achieved, the mean number of mice required to reject an inactive compound being 2·0.

4. The running component is highly sensitive to anticonvulsants and general central depressants, and insensitive to phenothiazine tranquillizers and morphine. Reserpine caused an increase in the severity of the running component.

5. The statistical model used in this test is of general application to screening test situations which use quantal data.

Audiogenic seizures in mice follow a pattern composed of a primary rapid running phase followed by clonic and tonic convulsions often ending in respiratory arrest which necessitates positive pressure ventilation. The important parameters influencing these seizures were reviewed by Bevan (1955).

The use of audiogenic seizures for evaluating a variety of centrally acting compounds was reviewed by Riley & Spinks (1958), and in all cases convulsions were used as the main positive criteria.

We have found that centrally acting drugs can be detected using the running phase only and we have made this the basis of a screening test because of its simplicity, reduction in losses due to convulsions and because detailed analysis of anticonvulsant action was not required.

This paper describes a sequential screening test suitable for the detection of central depressants, anticonvulsants and certain other compounds with miscellaneous activities. The test is clearly non-specific and was selected as a primary screen in

order to detect a wide range of centrally acting compounds in an economical manner with the understanding that detection would be followed by more specific procedures.

Initially, animals of the DBA/1 strain obtained from the Laboratory Animals Centre, Carshalton, were used, but they were unsatisfactory for screening purposes because of the small litter size coupled with a rather weak physical constitution. This problem was overcome by crossing DBA/1 males with Smith & Nephew Research strain (SNR) albino females and using the F_1 generation.

Screening was previously carried out by comparing several drug-treated groups with a saline-treated group and analysing the results using the Fisher-Yates exact probability test (Finney, 1948).

The control groups from these experiments totalled 1330 animals and these were used to calculate the running positive fraction described under the heading *Theoretical considerations.*

Methods

Animals

The mice used were the F_1 generation obtained by crossing DBA/1 males with SNR albino females.

The F_1 animals were of five colours, black 42·5%, brown 34·9%, agouti 15·8%, black/white 4·2% and white 2·7%. The average litter size was 6·4, in contrast to the DBA/1 with a litter size of 2·6.

Apparatus

The sound chamber consisted of a 33·7 cm diameter shallow cylindrical steel vessel with a circular vertical wall 19·1 cm high. An 8 V electric doorbell mounted on the inside at the top of the wall provided the stimulus which was 117 ± 2 db re $0{\cdot}2 \times 10^{-4}$ μ-bar. Measurements of sound pressure level were made using a Bruel and Kjaer 2604 amplifier and 4136 microphone.

A constant intensity of stimulation is important as it has been found that variations of sound pressure level significantly affect potency determinations (Horlington, unpublished observations).

The sound chamber was housed in a thick walled wooden box with a Perspex lid for observation.

The audiogenic seizure test was carried out in an air conditioned laboratory maintained at $21° \pm 1°$ C. The push button operating the bell simultaneously lit a 150 W bulb to illuminate the chamber.

Procedure

Mice were placed singly into the chamber and the bell sounded until either a positive response occurred or 20 sec had elapsed. A positive response consisted of extremely rapid running (pre-convulsive excitement), non-responsive mice usually remaining stationary (freezing).

If the bell push was released immediately after running started the animal made only two or three circles of the chamber before stopping; often running ceased immediately the stimulus ceased. A small percentage of animals continued to run

and then developed clonic, followed by tonic, seizures. The latter die from respiratory failure unless positive pressure respiration is applied with a tube and rubber bulb (A. Brown, personal communication).

The criterion for a positive response is the occurrence of a running response within 20 sec of sounding the bell. The average latency $\pm$ S.E.M. to onset of running was $7{\cdot}4 \pm 0{\cdot}28$ sec ($n = 100$).

Positive animals were selected in this way and allocated to drug treatment groups. Control groups treated intraperitoneally with saline (or untreated) exhibit 78% positive responses and this figure is the basis of the control graph (Fig. 1).

Susceptibility to audiogenic seizures

The F_1 generation has been investigated to find the relationship between audiogenic seizure susceptibility and the following factors, age, sex and coat colour.

The susceptibility to audiogenic seizures was not related to sex or coat colour. Susceptibility altered with age, however, 25% of the mice giving positive responses during the fifth and sixth week and subsequently the percentage was reduced.

The susceptibility for DBA/1 in this laboratory is 69% although we have not carried out a detailed study of the relationship with age for this strain.

PD50 determination for reference compounds

A group of animals which had positive responses was selected and from this, animals were allocated to different dose levels in groups of eight.

Drugs were administered either intraperitoneally or orally and 45 min later the mice were re-tested using a latin square design for inter-group randomization.

PD50s were determined and the 95% confidence limits calculated using the method of Litchfield & Wilcoxon (1949).

Drugs

The following drugs were used: acetazolamide sodium, atropine sulphate, chloral hydrate, chlordiazepoxide, chlorpromazine HCl, dichloralphenazone, ethosuximide, haloperidol, meprobamate, morphine hydrochloride, nitrazepam, orphenadrine citrate, phenobarbitone sodium, phenoxypropazine hydrogen maleate, phenytoin sodium, primidone, reserpine, trifluoperazine dihydrochloride, troxidone.

The drugs were dissolved in water, or in 0·9% saline or suspended in gum acacia 10% w/v in water. Doses of organic salts are expressed throughout as weights of active acid or base/kg body weight.

Theoretical considerations

Accumulated control data from 1,330 controls as described under the heading *Procedure* indicated the applicability of a binomial model. Unfortunately the number of mice used in each control experiment was not constant. However, the fit of the model can be examined by reference to Table 1.

If it can be assumed that a mouse in the test is selected at random from a population of mice containing a certain proportion P that will respond to the stimulus, and

that the mice respond independently of each other, then the number of mice responding to the sample tested has a binomial distribution with the probability of running for each mouse equal to P.

The actual numerical value of P is not crucial to the theory but is needed for the correct scaling of the P axis in the operating characteristic of the test (see below). Here the value of P is taken to be 0·78.

If the value of P varies during the tests, control groups would be necessary for each test and the operating characteristic would vary from test to test. However, if P remains stable during the tests, control groups are not necessary and the operating characteristic remains constant. This is discussed more fully later.

The object of the test is to distinguish, from a large number of compounds, those which are 100% effective in suppressing the running component in the whole population of mice. We define effectiveness by saying that if a compound is, for example, 60% effective, then after injection of the compound, the probability of running is 0·4, given that running would occur. It should be noted that some mice will not have seizures even though the compound is ineffective. It is therefore necessary to calculate the chance that a compound which is not very effective still produces 100% protection in the sample of mice tested.

Probability of seizure in test

Of the population of mice available for the tests, only a proportion (P) will exhibit running, even without drug protection. It is only this proportion which can be protected.

TABLE 1. *Observed and expected number of experiments for different running fractions based on a binomial model*

Observed fraction running	Observed No. of experiments	Theoretical fraction running out of ten mice	Expected No. of experiments
1·0	11	1·0	11·2
0·95–0·85	32	0·9	31·7
0·85–0·75	38	0·8	40·3
0·75–0·65	32	0·7	30·3
0·65–0·55	14	0·6	15·0
0·55–0·45	6	0·5	5·1
0·45–0·35	2	0·4	1·2
		0·3	0·2
Total	135	Total	135

The observed data are calculated from 135 experiments using 1,330 mice. The expected number of experiments was calculated assuming a binomial with P=0·78 and ten mice per experiment, the value for P being obtained from the overall control running fraction.

TABLE 2. *Probability of either a seizure or protection following treatment with compounds of differing effectiveness*

Effectiveness of compound (%)	100	95	90	80	50
Probability of seizure	0	0·039	0·078	0·156	0·390
Probability mouse will not respond	1·0	0·961	0·922	0·844	0·610

For example, taking P = 0·78, if the compound is 80% effective then 80% of this proportion is protected and only 20% will exhibit running. The proportion responding is, therefore, 20% of 0·78, that is 0·156.

The probability that a mouse will not respond is 1 – probability of seizure: 1 – 0·156 = 0·844 = 0·22 + 80% of 0·78. Further results are given in Table 2, and this table will be used in the calculation of the operating characteristic of the test.

Test criterion

It is required to accept only those compounds which are 100% effective in suppressing the running component because only those compounds which could achieve this were judged to be of further interest. The test criterion is therefore that any compound that does not induce complete suppression is rejected.

Errors

Since any compound which results in complete suppression of seizures is accepted and since, by definition, 100% effective compounds induce complete suppression, it is not possible to reject any compound which is 100% effective.

It is possible, however, that complete suppression is achieved even though the compound is not 100% effective. For example, suppose the compound is 90% effective, from Table 2, the probability of a seizure is seen to be 0·078. If the test uses ten mice, the probability of complete suppression (no seizures) is $(1–0·078)^{10}$, or 0·444. Therefore, 44·4% of a series of tests on this compound would lead to the acceptance of this compound as 100% effective. This error can be made as small as required by increasing the number of mice used in the test. For instance, using twenty mice reduces the above probability to 0·197.

The operating characteristic

The operating characteristic of the test is the probability of accepting the compound as 100% effective as the actual effectiveness varies.

For this test the operating characteristic depends only on the maximum number of mice used per compound and not on the sequence in which the mice are tested.

TABLE 3. *Operating characteristic-probability of accepting compounds of differing % effectiveness as 100% using different numbers of mice*

Number of mice used	Probability of acceptance as 100% effective. Actual compound % effectiveness				
	100	95	90	80	50
16	1·000	0·529	0·273	0·065	0 000
20	1·000	0·451	0·197	0·034	0 000
25	1·000	0·369	0·131	0·014	0 000

TABLE 4. *Number of mice used at each stage of the sequential test*

Stage	1	2	3	4	5	6	7
No mice/stage	1	1	2	2	2	4	4

Test procedure

It is inherent in the test criterion that a compound may be rejected if it fails to protect a single mouse and the sequential organization of the test is designed to take advantage of this to minimize the number of mice used per compound. By applying the audiogenic seizure test sequentially to single animals and rejecting the compound on the first occasion a mouse exhibits running, rejected compounds are eliminated at the earliest possible opportunity. However, because the screening test is to be applied to a large number of compounds, most of which will be eliminated in the early stages, it is convenient to combine the later stages by using two mice and later four mice simultaneously as shown in Table 4.

This has the advantage of reducing the total time to screen all compounds under test. The combination of the later stages, however, does mean that the number of mice is not the minimum possible, but it is reasonably obvious that if most of the compounds are eliminated in the early stages, the additional number of mice used is small. Reference to the results in Table 5 will clarify these points.

At Stage 1, a single positive animal, selected on the day of the test, is injected intraperitoneally with the drug at 1/5 LD50 and 45 min later exposed once more to the bell. If the mouse exhibits running the compound is rejected as inactive at Stage 1, and if the mouse does not run the compound moves to Stage 2.

From experience with this test, it was found that compounds reaching $n=16$ protected mice were of a sufficient interest for further evaluation to be carried out and in view of this the sequence of stages shown in Table 4 ends with a total of sixteen mice.

Controls

For each set of controls the number of animals responding, r, is recorded and the deviation $r - 0{\cdot}78n$ was calculated where n was the number of animals used. In the present experiments n was always 4. This is cumulated with the deviation already recorded from previous controls and the cumulative deviation plotted against the cumulative total of all controls.

If the proportion responding is stable at 0·78, the plot should be essentially horizontal. A rising plot would indicate a higher proportion responding and a falling plot a lower proportion. However, it is necessary to decide whether an observed change from the horizontal is really due to a change in proportion responding or whether it is simply a short term fluctuation due to the sampling effect. With a sample of size 4, this sampling effect can be quite large because in selecting four mice from a population of which 78% respond, there is a reasonable chance of observing no, one, two, three or four responding mice.

The decision is made using a mask as shown in Fig. 1.

The procedure consists of placing the mask on the plot with the centre point touching the last point plotted and with the rectangular boundaries parallel to the areas of the chart.

If the plot lies wholly within the arms of the mask (produced if necessary) the proportion responding is in control. However, if the arms of the mask intersect the plot, the data are considered as providing evidence that the proportion responding has changed. Intersection of the lower arm indicates an increase, and of the upper arm, a decrease, in proportion responding.

The dimensions of the mask are determined by the two angles A_1 and A_2 and by the two distances d_1 and d_2. The theory underlying the test is reviewed by Johnson & Leone (1962a, b, c). The application to the binomial distribution (Johnson & Leone, 1962c) uses a plot of cumulative number responding against cumulative number sampled. The modification to apply the theory to the plot of cumulative deviation from expected number responding is mentioned towards the end of the paper and consists essentially of choosing a new pair of axes. The cumulative number sampled axis is taken along the line of cumulative expected number

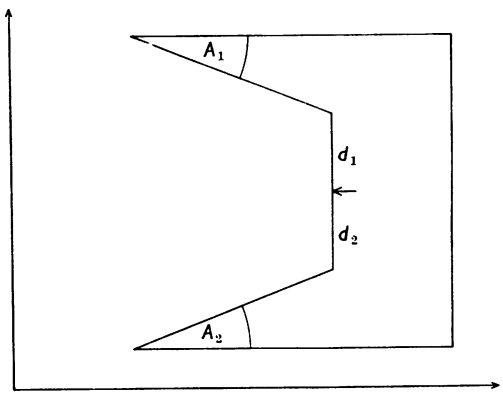

FIG. 1. Mask for cumulative sum control chart. The mask in this figure is a theoretical example illustrating the dimensions involved in the calculations shown in **Appendix**.

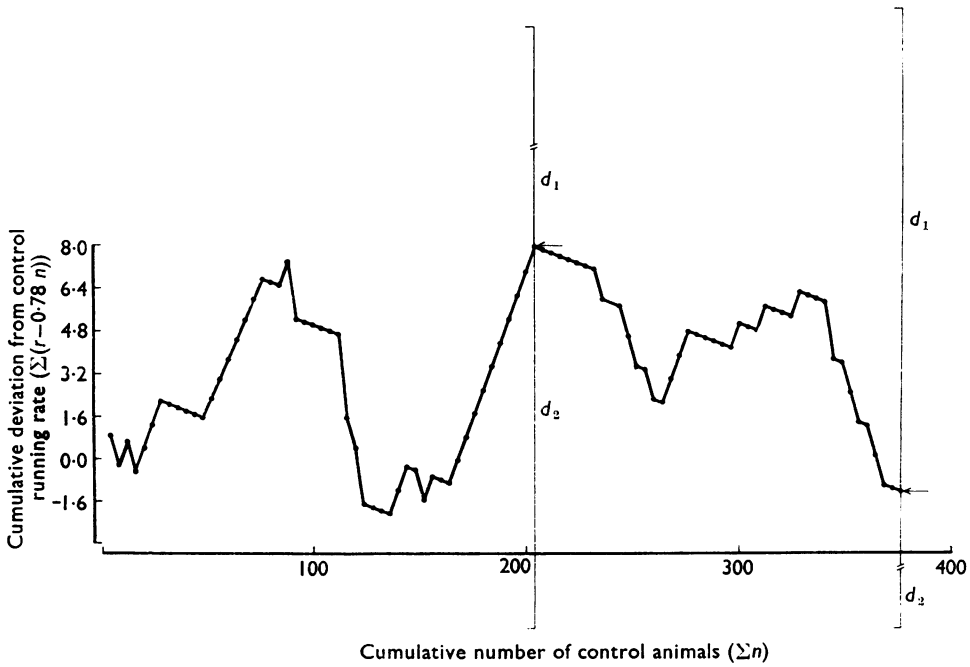

FIG. 2. Cumulative sum control chart. r=Number of animals responding, n=control sample size. Lines d_1 and d_2 are arms of the control mask fitted at the last point and at the point of maximum deviation. The mask has become a horizontal slot because the angles A_1 and A_2 of Fig. 1 are almost zero for these data.

responding. The new vertical axis now measures the cumulative deviation from expected number responding. The mask is not altered, but it is now convenient to specify its dimensions by angles referred to the new horizontal axes and by distances measured perpendicular to this new axis. The values of these are calculated by simple geometry.

Figure 2 shows the application of the mask (as calculated in the **Appendix**), to the final point and also to the earlier point showing the greatest deviation. Note that since the angles A_1 and A_2 are almost zero, the mask has degenerated into a horizontal slot.

Four control mice were tested for running on each day the screening test was applied until it became clear that the proportion running in the mice to be used in the test was stable at $P=0{\cdot}78$.

Results

Screening tests

Two hundred and forty-two new compounds representing a variety of chemical structures were screened, and from these eleven were accepted as positive having protected sixteen animals at 1/5 LD50.

TABLE 5. *Percentage of compounds rejected at each stage of the sequential test*

Stage No.	Cumulative number of animals used	% of compounds rejected
1	1	63·2
2	2	19·0
3	4	11·3
4	6	4·8
5	8	1·3
6	12	0·4
7	16	0·0

TABLE 6. *Potencies (PD50s) and LD50/PD100 ratios of some centrally acting drugs against the running component of audiogenic seizures*

Drug	Route	PD50 (mg/kg)	95% fiducial limits	LD50 (mg/kg)	PD100 (mg/kg)	LD50/PD100 ratio
Anticonvulsants						
Acetazolamide sodium	i.p.	9·1	4·9–16·4	3000	36·2	75·0
Ethosuximide	i.p.	146·0	110·5–192·5	1372	250·0	5·5
Phenobarbitone sodium	i.p.	2·3	1·6–3·4	340	7·3	42·5
Phenytoin sodium	i.p.	13·9	7·7–25·0	184	45·9	4·0
Primidone	i.p.	1·3	0·7–2·3	1060	6·0	177·0
Troxidone	i.p.	142·0	103–195	1800	230·0	7·8
Central depressants						
Chloralhydrate	p.o.	47·0	32–71	1116	150·0	7·4
Chlordiazepoxide	i.p.	1·95	1·4–2·7	268	5·0	53·6
Dichloralphenazone	p.o.	52·0	35–79	1370	170·0	8·1
Nitrazepam	i.p.	0·14	0·08–0·3	275	1·4	196·0
Haloperidol	i.p.	0·75	0·39–1·42	60	5·0	12·0
Meprobamate	i.p.	29·0	18–47	710	125·0	5·7
Sodium γ-hydroxybutyrate	i.p.	167·5	126·8–221·1	>2000	350·0	>5·7
Miscellaneous						
Atropine sulphate	i.p.	41·6	—	208	833·0	0·25
Phenoxypropazine hydrogen maleate (MAOI)	i.p.	15·8	11·7–21·3	330	42·0	7·9
Orphenadrine citrate	i.p.	11·8	6·4–21·7	75·8	28·1	2·7

PD100 values were obtained from graphs drawn for PD50 estimations. LD50 values were obtained from the literature, directly from manufacturers, or by determination at SNR using albino male mice.

For the remaining two hundred and thirty-one compounds, it will be seen from Table 5 that the bulk of these were eliminated in the first two stages.

Four hundred and thirty-nine animals were used in rejecting compounds, a mean of 2·0 per compound which is a considerable improvement on our previous method which used not less than six to achieve the same result.

PD50s

For the sake of simplicity a rather heterogenous group of compounds has been placed under a single description, " central depressants " in Table 6.

Table 6 lists the PD50 values for a number of reference compounds and also the ratio between the PD100 and LD50 which is a measure of the risk of non-acceptance by the sequential screening test. These compounds caused minimal, or in most cases, no observable behavioural effects in the PD50 dose range. Chlorpromazine HCl and morphine HCl gave erratic results without a dose response relationship below doses causing gross locomotor incapacitation and are classed as inactive.

A dose response relationship was obtained with trifluoperazine dihydrochloride and a PD50 of 11·5 mg/kg intraperitoneally obtained, but gross behavioural effects including prostration occurred at this dose level.

Reserpine caused an increase in the severity of the convulsions.

Discussion

One feature of the screening test which offers an opportunity for accidental rejection of a useful compound is the 100% effectiveness criterion, because a single mis-injection could reject an active compound.

Another feature involving a rejection risk is the use of a single dose level based on a fixed ratio of the LD50. The LD50/PD100 ratio of a compound, which is a form of therapeutic index, influences its performance in the test, and with a screening dose of 1/5 LD50, if the ratio is less than 5 the compound is likely to be rejected. This applies to three of the reference compounds in Table 6, of which phenytoin sodium is the most worrying. Raising the dose above 1/5 LD50 to avoid this would, however, lead to an increase in the numbers of compounds accepted, and reduce the rigor of the test below the required level. At the other end of the scale, nitrazepam with a ratio of 196 is extremely unlikely to be rejected.

With regard to the potency of reference compounds, Swinyard, Castellion, Fink & Goodman (1963), using Swiss albinos, found the tonic extension component to be more sensitive than the running component. We do not have data on tonic extension, but the PD50 values shown in Table 6 may be compared with the PD50 values for tonic extension obtained by Swinyard *et al.* (1963) which were troxidone 375 (326–431), phenobarbitone 4·6 (2·1–6·9), phenytoin 5·0 (3·3–7·5) and meprobamate 29 (21·8–38·6). The result for ethosuximide in our test is of interest because this drug has been shown to act against metrazole convulsions in rats but not against electroshock in mice (Chen, Weston & Bratton, 1963), indicating that the running component is suitable for the detection of this type of compound.

The sensitivity to general central depressants is high, the most useful example being chloral hydrate, since, in other tests, such as the rotor rod test, doses of between 200 and 400 mg/kg are required to give 50% effects.

Our results with chlorpromazine and reserpine are in agreement with those of Swinyard *et al.* (1963) and Fink & Swinyard (1960), who used Swiss albino mice singly.

Plotnikoff (1960) stimulating groups of five mice confirmed the above results for chlorpromazine using Swiss albinos, but obtained protection with a PD50 of 4·8 mg/kg intraperitoneally when using DBA/1 mice. With reserpine, he obtained protection in both strains, the PD50 values being 5·0 mg/kg (DBA/1) and 9·0 (Swiss albinos).

Another anomaly is that large oral doses of phenobarbitone (100 mg/kg) used by Plotnikoff (1958) did not inhibit running, and even caused potentiation in Swiss albino mice. We have found no difference between DBA/1 and the hybrid with regard to their responses to reserpine, chlorpromazine or phenobarbitone. Plotnikoff (1963) expressed the view that anomalies between his results and those of Fink & Swinyard (1959) were due to the use of grouped rather than single mice, but this suggestion does not seem to have been investigated further.

Appendix

Cusum control chart

Notation

P_o = the expected proportion responding.

P = the alternative value of the proportion responding it is considered essential to detect.

α = the probability that the scheme will wrongly indicate a change in proportion responding from P_o to P.

ARL = the average run length—the average number of observations before an "out of control" point is observed.

Dimensions of the mask

The mask is so constructed that if the proportion responding remains stable at P_o, the ARL is very high, but if the proportion responding changes to P, the ARL is low so that the change is soon detected.

Calculate: (i) $a=\log [(1-P_o)/(1-P)]$; $b=\log [P_1/P_o]$; $c=\log \alpha$

(ii) scaling factor K (to take account of the scaling used in the plot). If one unit of length on the chart represents x units of cumulative number sampled and y units of cumulative deviation then, $K=x/y$.

(iii) $\tan \sigma_o=KP_o$; $\tan \sigma=Ka/(a+b)$ and hence σ_o and σ

(iv) $A=|\sigma-\sigma_o|$, that is, the numerical value of the difference

(v) $d=|c \sin \sigma/a \cos A|$.

The calculations are repeated for two values of P; $P_1<P_o$ giving A_1 and d_1 and $P_2>P_o$ giving A_2 and d_2.

Calculations

In this application, $P_o=0{\cdot}78$, $\alpha=0{\cdot}025$, $K=12{\cdot}5$.

When $P=P_1=0{\cdot}73$ and using common logarithms

$a=1{\cdot}9111=-0{\cdot}0889$, $b=\bar{1}{\cdot}9712=-0{\cdot}0288$, $c=\bar{2}{\cdot}3979=-1{\cdot}6021$ $\tan \sigma_o=9{\cdot}7500$,

tan $\sigma = 9{\cdot}4447$ giving $\sigma_o = 84°8{\cdot}6'$, $\sigma = 83°57{\cdot}4'$.
Hence $A_1 = 0°11{\cdot}2'$, $d_1 = 17{\cdot}91$.
When $P = P_2 = 0{\cdot}83$
$a = 0{\cdot}1120$, $b = 0{\cdot}0270$, $c = \bar{2}{\cdot}3979 = -1{\cdot}6021$
tan $\sigma = 10{\cdot}0727$ giving $\sigma = 84°19{\cdot}8'$
Hence $A_2 = 0°11{\cdot}2'$, $d_2 = 14{\cdot}24$.

Application

The angles A may be taken as zero so that the mask degenerates to a horizontal slot and the test is shown in Fig. 2.

As is shown by the figure, the plot of the control mice shows no evidence against the hypothesis that $P = 0{\cdot}78$.

ARL

The ARL for P is calculated by the formula $|c/[a(1-P)+bP]|$. For both $P_1 = 0{\cdot}73$ and $P_2 = 0{\cdot}83$ the ARL is less than 40. This means that the average number of samples before a change to P_1 or P_2 was detected, is less than 10.

Note that this method does not require the sample sizes to be equal although in this case they were.

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