# OBSERVATIONS ON THE USE OF A MOUSE BIOASSAY METHOD FOR INVESTIGATING PURGATIVE ACTIVITY

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Received April 12, 1962

Some essentially practical observations have been made on the use of a simple purgative assay in mice. Modifications of the method have improved both the accuracy and precision of the assay. Senna extracts have been assayed in terms of a sennoside A standard.

The mouse is well suited for investigating the purgative activity of some anthraquinone drugs (Geiger, 1940; Collier, Fieller and Paris, 1948; Lou, 1949; Fairbairn, 1959). Moreover, McClure Browne, Edmunds, Fairbairn and Reid (1957) have shown that the clinical results obtained with senna preparations are in good agreement with the purgative activity predicted from the mouse assay method described by Lou (1949). In this present work, the latter method of assay has been systematically examined to improve its inherent accuracy and precision. Furthermore, an attempt has been made to use the mouse method of assay for routine standardisation of senna extracts in terms of sennoside A as a reference standard.

## Materials and Methods

### *Materials*

Throughout this work two powdered extracts of Alexandrian senna pod (S.1, S.2) were used; immediately before use these extracts were suspended in distilled water. A sample of sennoside A (prepared in the Pharmaceutical Research Department, Allen & Hanburys Limited) was used as the laboratory standard. Solutions of this material were prepared in distilled water to which trace amounts of sodium bicarbonate were added.

### *Methods*

The basic method of assay used is that described by Lou (1949), although subsequent modifications have been made. At the commencement of this work, it was thought that the following procedure would be suitable for assaying senna extracts with a reasonable degree of accuracy and precision.

Male albino mice of body weight 18 to 22 g. are conditioned by being subjected at weekly intervals to a "dummy" assay procedure, involving starving, dosing with a purgative and placing in cages, since D'Arcy, Grimshaw and Fairbairn (1960) and D'Arcy (1962) have shown that the initial training of the animals improves the precision of the assay. After this initial training period, which may take from 3 to 4 weeks, the mice are used routinely at weekly or fortnightly intervals. Food, but not water, is removed 2 hr. before the assay and during this time the mice are isolated

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in perspex cages placed on wire grids over blotting paper; if any animal should show evidence of diarrhoea it is excluded from the test.

The animals are then randomised in groups of 10, and solutions or suspensions of standard and test materials are prepared immediately before use and administered orally in a dose volume of 0·5 ml./mouse, regardless of body weight. Depending upon the material being assayed, either 2 or 3 dose groups are used for the standard and each test preparation. After dosage the mice are returned to the perspex cages, two mice per compartment. The compartments are 5 × 3½ × 7 in. high, open top and bottom and are built of perspex in blocks of 10. They stand on ¼ in. mesh wire grids raised ½ in. above sheets of blotting paper, the cage tops are also covered with grids. A small container, filled with a mixture of 10 parts crushed cubes (Diet 41) to 7 parts water, is placed in each compartment 6 hr. after the start of the assay.

At 3, 6 and 22 hr. from the start of the assay the grids and blotting papers are changed, and the number of unformed faeces counted for each pair of mice. Unformed faeces are wet, shapeless, relatively large in size and stain the blotting paper. The total unformed faeces excreted in 22 hr. by pairs of mice within each dose group is used as the response metameter. The potency ratio and fiducial limits of the assay are calculated by standard statistical methods.

## RESULTS

### *Dose Response Relationships*

*Response-time.* Purging of mice can be induced by oral doses of 0·5 to 2·5 mg. sennoside A/mouse and 5 to 30 mg. of the senna extracts/mouse. Initial experiments indicated that the total number of wet faeces excreted by pairs of mice, during 22 hr. after drug administration, provided a satisfactory response metameter. To test whether the purgative activity was complete by 22 hr., three experiments were made in which senna extract (S.1) was assayed against sennoside A, the total number of unformed faeces being counted at 3, 6, 22, 25 and 28 hr. Table I summarises the cumulative responses for each dose; responses from the three separate assays have been summed.

TABLE I

SUMMED CUMULATIVE RESPONSES FOR THREE ASSAYS OF SENNA EXTRACT (S.1) AGAINST SENNOSIDE A

| Sample | Dose mg./mouse orally | No. of unformed faeces* excreted; time after dosage | | | | |
|---|---|---|---|---|---|---|
| | | 3 hr. | 6 hr. | 22 hr. | 25 hr. | 28 hr. |
| Senna extract S.1 .. .. | 10 | 10 | 36 | 43 | 44 | 44 |
| | 20 | 26 | 65 | 88 | 89 | 89 |
| Sennoside A .. .. .. | 0·75 | 12 | 32 | 47 | 47 | 47 |
| | 1·5 | 15 | 60 | 84 | 86 | 87 |

* Each figure is the cumulative total, for 5 pairs of mice, summed over three assays.

The results show that, for both sennoside A and the senna extract (S.1), the responses were substantially complete by 22 hr., thus confirming that this was a suitable time at which to terminate an assay.

*Dose-response curves.* Using the cumulative responses at 22 hr., a log dose-response curve was established for sennoside A. The responses produced by doses of 0·5 to 2·5 mg. sennoside A/mouse lay on the rectilinear portion of the curve. In some experiments, sennoside A was administered at higher dosage, i.e., 3·0 mg./mouse; however, this dose was not suitable for assay use since it produced severe purging and prostration.

Dose-response curves were similarly established for the two senna extracts (S.1, S.2) and were linear within the range 7·5 to 30 mg. extract/mouse. The dose-response curve for each extract did not deviate significantly in parallelism ($P > 0{\cdot}05$) from that of sennoside A.

*Housing of Animals During the Assay Procedure*

Two dose-response curves were established for sennoside A using 10 mice per dose group. In the first series of experiments the animals were housed separately and their unformed faeces counted over a 22 hr. period. Secondly, mice were housed in pairs and the responses measured as previously described. The results of these experiments (Table II) show that there is no gain in precision to be obtained by housing the mice singly; in subsequent experiments the mice were therefore caged in pairs.

TABLE II

EFFECT OF HOUSING MICE EITHER SINGLY OR IN PAIRS ON THE PRECISION OF THE DOSE-RESPONSE CURVE FOR SENNOSIDE A

| Statistic | Animals housed singly | Animals housed in pairs |
|---|---|---|
| b | 6·5 | 11·5 |
| $s.e._b$ | 0·47 | 0·77 |
| $b/s.e._b$ | 13·83 | 14·93 |

*Effects of Dose Volume and Adjustment for Body Weight*

*Effect of dose volume.* The effect of variation in dose volume on the precision of the assay was studied. Three groups of 24 mice were used and they were dosed with volumes of either 0·25, 0·5 or 1·0 ml./mouse. For each of the three groups a dose-response curve was established using 8 mice per dosage group at levels of 0·5, 1·0 and 2·0 mg./mouse of sennoside A. The results of this experiment and their analysis are presented in Table III. It is evident that, in all three dose-volume groups, the response to sennoside A was linear over the dose range studied, since the sums of squares attributed to both "combined curvature" and "opposed curvature", were not significant. In addition, there was no significant difference in the level of responses to a specific dose of sennoside within each dose-volume group, since the "between dose-volume" term was not significant. Furthermore, as the "parallelism" term was also not significant, there was no difference between the slopes of the dose-response curves within the three dose-volume groups. However, the "within dose error" for the 0·5 ml. dose volume group was less than that of the other two groups.

TABLE III

THE EFFECT OF DOSE VOLUME ON ASSAY PRECISION

Assay protocols

| Dose Volume ml./mouse orally | Dose of sennoside A mg./mouse | No. of unformed faeces: Cumulative 22 hr. response per pair of mice | | | | Total response |
|---|---|---|---|---|---|---|
| 0·25 | 0·5 | 3, | 0, | 1, | 4 | 8 |
| | 1·0 | 6, | 7, | 3, | 5 | 21 |
| | 2·0 | 10, | 9, | 9, | 9 | 37 |
| 0·5 | 0·5 | 1, | 1, | 3, | 2 | 7 |
| | 1·0 | 6, | 2, | 7, | 6 | 21 |
| | 2·0 | 10, | 10, | 10, | 10 | 40 |
| 1·0 | 0·5 | 1, | 4, | 2, | 4 | 11 |
| | 1·0 | 5, | 9, | 3, | 5 | 22 |
| | 2·0 | 8, | 9, | 8, | 11 | 36 |

Analysis of Variance

| Source | S.S. | d.f. | M.S. | F. |
|---|---|---|---|---|
| Between dose volumes | 0·389 | 2 | 0·195 | <1 |
| Linear regression | 315·375 | 1 | 315·375 | 123·87* |
| Parallelism | 4·000 | 2 | 2·000 | <1 |
| Combined curvature | 1·681 | 1 | 1·681 | <1 |
| Opposed curvature | 0·111 | 2 | 0·056 | <1 |
| Total between doses | 321·556 | 8 | | |
| Within dose error (Volume 0·25 ml.) | 19·500 | 9 | 2·546 | |
| Within dose error (Volume 0·5 ml.) | 17·500 (68·750) | 9 | | |
| Within dose error (Volume 1·0 ml.) | 31·750 | 9 | | |
| Total | 390·306 | 35 | | |

* Very highly significant.

*Adjustment of dosage for body weight.* In order to examine the effect of dosing per body weight or per mouse, it was necessary to house the animals individually. Solutions of 2 or 4 mg. sennoside A/ml. were administered to groups of 10 mice of body weight 27 to 34 g. In dosing, a volume of 0·5 ml./30 g. was used; in dosing irrespective of body weight, a volume of 0·5 ml./mouse was given. Results are shown in Table IV; there was no difference in level of response which ever method of dosage was used, nor was any gain in precision obtained by dosing on a bodyweight basis.

*Effects of Training on Accuracy and Precision*

The effect of the training procedure on the accuracy and precision of the assay method has been correlated with age and weight of the animals. The purgative activity of senna extract (S.2) was assayed against sennoside A using a 2 × 2 design, at weekly intervals for 4 weeks and subsequently at fortnightly intervals. For each assay the mean body weight of the animals was determined; the dose volume administered was 0·5 ml./mouse regardless of body weight; animals were housed in pairs, 5 pairs per dose group. The results of these experiments (Table V) show that the best accuracy and precision was obtained in the fifth week of training. However, since the mice increased in weight during the training period it was deemed necessary to investigate the effect of weight alone. Therefore,

the purgative activity of senna extract (S.2) was again assayed against sennoside A using, for each separate assay, groups of mice within a narrow body weight range. In five assays (Table VI), the mean weight of the mice varied from 18·8 to 36·8 g., all mice being untrained.

TABLE IV

THE EFFECT OF ADJUSTMENT OF DOSE VOLUME FOR BODY WEIGHT ON ASSAY PRECISION

Assay protocols

| Mean weight g. ± s.d.* | Dose Sennoside A | | No. of unformed faeces | |
|---|---|---|---|---|
| | mg./ml. | Dosage | Cumulative 22 hr. response per mouse | Total response |
| 30·3 ± 1·70 | 4·0 | 0·5 ml./30 g. | 5, 5, 8, 4, 3, 6, 5, 5, 5, 4 | 50 |
| 29·5 ± 1·51 | 4·0 | 0·5 ml./mouse | 5, 4, 3, 4, 2, 4, 6, 4, 6, 6 | 44 |
| 30·8 ± 2·15 | 2·0 | 0·5 ml./30 g. | 5, 0, 3, 4, 1, 1, 5, 4, 5, 2 | 30 |
| 29·8 ± 2·30 | 2·0 | 0·5 ml./mouse | 2, 6, 4, 2, 3, 1, 2, 4, 3, 4 | 31 |

* Standard deviation (s.d.) has been tabulated since it provides the best measure of scatter between the body weights.

Analysis of variance

| Source | S.S. | d.f. | M.S. | F. |
|---|---|---|---|---|
| Between methods of dosage | 0·625 | 1 | 0·625 | <1·0 |
| Linear regression | 27·225 | 1 | 27·225 | 11·77† |
| Parallelism | 1·225 | 1 | 1·225 | <1·0 |
| Total between doses | 29·075 | 3 | | |
| Error 1 (0·5 ml./30 g.) | 48·0 | 18 | } 2·313 | |
| Error 2 (0·5 ml./mouse) | 35·333 | 18 | | |
| Total | 112·375 | 39 | | |

† Highly significant.

The results indicate that accuracy and precision in the untrained mice is satisfactory when their mean body weight lies between 27 to 31 g. Although accuracy and precision are improved to some extent by using heavier animals, comparison with the previous assay data (Table V) indicates that the degree by which these indices are improved is greater with trained heavier mice. In current assays, the use of mice, initially weighing 25 to 30 g. and subsequently conditioned for 2 weeks, gives good results.

## DISCUSSION

The method of determining the purgative activity of senna extracts using mice is relatively simple and easy to perform, and in our experience and in the hands of others (Fairbairn, 1958) it has proved reliable and repeatable. Although the method we have described has been successful for the examination of senna, senna extracts and senna preparations, it is not so well suited to the evaluation of the activity of other anthraquinone purgatives, for example, cascara and rhubarb. The method does not demonstrate the purgative activity of aloin and phenolphthalein (D'Arcy, Grimshaw and Fairbairn, 1960).

The selection of sennoside A as a reference standard has provided a single chemical compound, in terms of which, the purgative activity of senna extracts can be evaluated, since in our experience dose-response curves for senna extracts are parallel to that of the standard sennoside. Although we have observed that the rates of onset of activity of the extracts and sennoside A differ, purgative activity is complete after 22 hr. for both extracts and sennoside A. Calculation of assay results over this period therefore provides a valid basis for assay.

TABLE V

THE EFFECT OF TRAINING ON THE ACCURACY AND PRECISION OF THE ASSAY OF SENNA EXTRACT (S.2) AGAINST SENNOSIDE A

| Week of training | Mean weight g. ± s.d.* | Known relative potency† | Estimated relative potency | 95 per cent fiducial limits (per cent) | λ = $s/b$ |
|---|---|---|---|---|---|
| 0 | 20·1 ± 1·2 | 0·25 | Assay | invalid‡ | |
| 1 | 24·9 ± 1·8 | 0·25 | 0·23 | 62–161 | 0·21 |
| 2 | 28·2 ± 2·3 | 0·25 | 0·19 | 61–164 | 0·19 |
| 3 | 30·1 ± 2·8 | 0·25 | Assay | invalid‡ | |
| 5 | 31·8 ± 4·0 | 0·25 | 0·24 | 76–131 | 0·12 |
| 7 | 34·8 ± 2·8 | 0·25 | 0·26 | 74–136 | 0·14 |
| 9 | 36·4 ± 6·0 | 0·25 | 0·24 | 65–153 | 0·19 |
| 11 | 38·0 ± 4·3 | 0·25 | 0·20 | 68–146 | 0·20 |

* As for Table IV.
† Mean of 10 assays using conditioned mice. 95 per cent fiducial limits of the mean 95–105 per cent.
‡ Dose response lines of test and standard deviated significantly from parallelism.

The potency of senna extract relative to sennoside A is the same whether mice are housed singly or in pairs during the assay. When low doses of purgative are administered to animals housed singly, the percentage of zero responses is large and their subsequent statistical treatment is questionable. However, by housing animals in pairs, and taking the sum of responses from the two animals, zero responses can be almost eliminated.

TABLE VI

THE EFFECT OF BODY WEIGHT ON THE ACCURACY AND PRECISION OF THE ASSAY OF SENNA EXTRACT (S.2) AGAINST SENNOSIDE A

| Mean weight g. ± s.d.* | Known relative potency† | Estimated relative potency | 95 per cent fiducial limits (per cent) | λ = $s/b$ |
|---|---|---|---|---|
| 18·8 ± 1·1 | 0·25 | 0·26 | 62–162 | 0·22 |
| 25·3 ± 1·9 | 0·25 | 0·16 | 68–147 | 0·15 |
| 27·5 ± 1·7 | 0·25 | 0·23 | 66–151 | 0·17 |
| 31·4 ± 3·7 | 0·25 | 0·25 | 70–143 | 0·16 |
| 36·8 ± 4·0 | 0·25 | 0·17 | 50–200 | 0·28 |

* As for Table IV.
† As for Table V.

In considering the effects of dose volume and adjustment for body weight on the accuracy and precision of the assay, the optimal dosage was found to be 0·5 ml./mouse regardless of body weight. Since, in practice, it is quicker and far more convenient to dose irrespective of body weight, this procedure is entirely suitable for routine work.

Conditioning of the mice before routine purgative testing greatly improves the assay. Initial studies, using mice 18 to 22 g. body weight,

showed that the best accuracy and precision was attained after a 5-week training period. In addition, later work using untrained animals, showed that when heavier mice were used, results were optimal with a weight range of 27 to 31 g. Thus it would seem that both the actual conditioning of the mouse to the test procedure, and the gain in weight during this training period influence the accuracy and precision of the assay. Current work using trained heavier mice has amply confirmed this observation.

When mice are fully conditioned, the assay procedure using 5 pairs of mice per dose group routinely gives 95 per cent fiducial limits of 75 to 130 per cent. This is satisfactory for routine estimations of purgative activity of senna extracts. Greater precision can be achieved by either using larger numbers of animals per dose group in individual assays or alternatively combining the results of two or more separate assays. The simple modifications that have been described have resulted in an increase in both the accuracy and the precision of the assay method.

*Acknowledgements.* We would like to thank Professor J. W. Fairbairn, School of Pharmacy, University of London, for his helpful advice, and Mrs. P. L. Hicks and Miss M. Kendall for technical assistance.

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