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Quantitative tear lysozyme assay: a new technique for transporting specimens

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SUMMARY We have developed a method for assaying the concentration of tear lysozyme using eluates of tear fluid collected on filter paper discs. Specimens can be stored and transported to remote laboratories for assay. We have shown that the 'indirect' or eluate method gives statistically comparable results to the 'direct' method using fresh, neat tear fluid.

We have previously described a tear lysozyme assay using tear fluid collected on filter paper discs.[1] Discs must be placed as soon as possible directly on to an agar plate seeded with *Micrococcus luteus* (*lysodeikticus*). The results of the assay have given a reliable guide to lacrimal gland function.[2] However, the organisation of the test has virtually required that the patient attends the laboratory. This has been inconvenient and has often led to patients travelling long distances.

Hypher[3] has criticised the test suggesting that a small amount of the tear fluid collected on to the disc remains in the bottle. He has calculated a correction factor for this.

We have now developed a modified test that allows the collection of tear fluid at a site distant from the laboratory and which minimises any loss of tear fluid from the disc.

Materials and methods

COLLECTION OF TEAR FLUID

Tear fluid was collected on to Whatman No. 1 filter paper discs, 5 mm in diameter, which were placed in the lower conjunctival sac. The volume of tear fluid collected was ascertained by weighing the disc in its bottle before and after collection. For this purpose a portable CAHN* automatic electrobalance was chosen (Fig. 1), as it could be set up on a desk in an outpatient clinic. It was accurate to +/- 0·01 mg equivalent to +/- 0·01 μl tear fluid.

A Hewlett-Packard printing calculator (Model 97S1/0) was used, as it was compatible with the output of the balance and could be programmed to calculate and print results of weight readings. Since no bottles were available commercially that were sufficiently small (maximum height 15 mm) or light (maximum weight 1500 mg), our Hospital Physics Department manufactured bottles by injection moulding from polypropylene beads.[4] A tool-steel mould was made with brass inserts that produced the bottle and lid as a one-piece moulding† (Fig. 2). Each bottle, with lid closed, weighed approximately 300 mg, measured 10 mm high by 11 mm wide and held up to 200 μl fluid. The bottles were cleaned in an ultrasonic water bath and dried in a hot air oven; a filter paper disc was then placed in each bottle. They were sterilised in batches by 2·5 megarads of irradiation.‡

PREPARATION OF ELUATE

After the bottle containing the tear fluid collected on to the disc had been weighed approximately 100 μl of phosphate-buffered saline was added from an Oxford pipette with a disposable tip and the lid was fastened. The bottle was reweighed to ascertain the exact volume of buffer added and the result recorded. After collection the bottles were transported at atmospheric temperature and placed overnight in a +4°C refrigerator. Next day they were transferred to a -20°C freezer in the laboratory pending assay.

*Cahn Instruments, Ventron Corporation, 16207 South Carmenita Road, Cerritos, California 90701, USA. Available in UK with Hewlett-Packard printing calculator and R51 balance from Oertling Instruments Ltd, Orpington, Kent.

†Manumold Mark 2 Injection Moulding Press, Florin Street, 457 Caledonian Road, London N7.

‡Irradiated Products Ltd, Elgin Drive, Swindon, Wilts.

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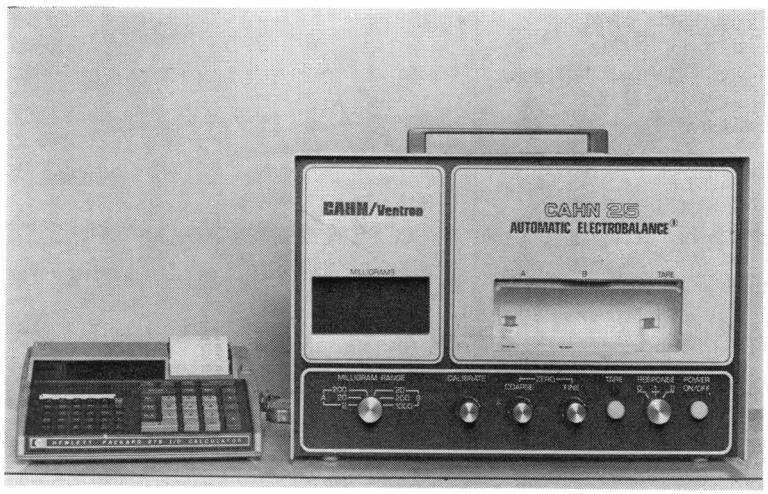

Fig. 1 *Portable electrobalance with printing calculator.*

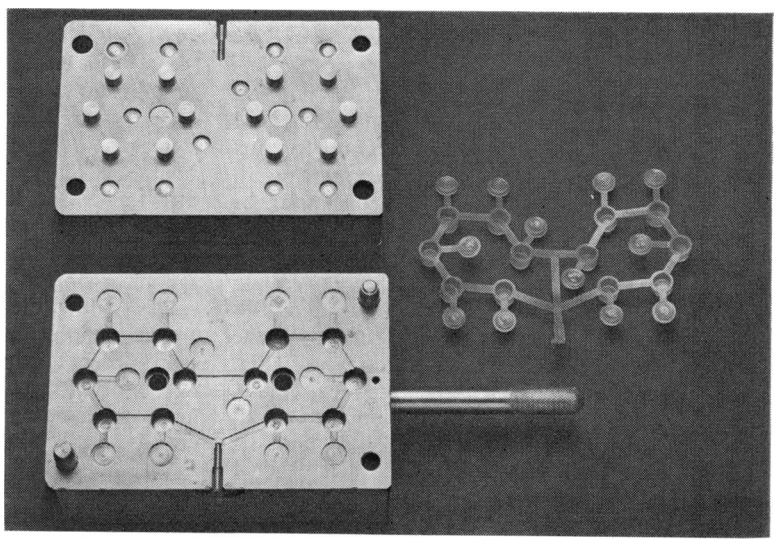

Fig. 2 *Injection mould and polypropylene bottles.*

PERFORMANCE OF ASSAY

For the assay, similar filter paper discs were prepared in 10×25 mm plastic tubes,* each tube being weighed on an Oertling R51 laboratory balance, accurate to +/- 0·01 mg. With the use of forceps 2 discs were dipped separately into the eluate of a tear fluid sample, weighed, and the volume taken up by each disc calculated. Batches of two discs were similarly dipped into 5 standards of hen egg lysozyme† containing 0·05, 0·1, 0·2, 0·3, and 0·4 mg/ml respectively, which were calibrated into units of activity per gram by our original spectrophotometric method.[1] All the discs were placed on an agar plate, previously flooded with *Micrococcus luteus* (*lysodeikticus*) and incubated at 20°C for 4 days. Zone diameters of bacterial lysis around the discs were measured on an image intensifier and recorded. After assay the eluates were frozen and stored at -20°C.

EVALUATION OF ASSAY

This new 'indirect' method of lysozyme assay was evaluated by sampling tear fluid from the right and left eyes of 42 'normal patients' (that is, patients with glaucoma but with normal lacrimal function clinically) aged from 20 to 79 years, with a mean of 56 years. These results were compared with those obtained by our original 'direct' method from 86 normal volunteers, aged 20 to 85 years, with a

*Metal Box Ltd, 30 Great Guildford Street, London SE1.

†Koch-Light Ltd, Colnbrook, Buckinghamshire.

mean of 50 years. Eight patients with established dry eyes, aged 9 to 70 years, were also tested by the 'indirect' method, and the results were compared with 23 patients with keratoconjunctivitis sicca, aged 42 to 68 years, tested by the 'direct' method.

TEST FOR ANTIBACTERIAL SUBSTANCES

Tear fluid was tested for the presence of antibacterial proteins other than lysozyme; which could theoretically inhibit the growth of *Micrococcus luteus*. Agarose containing antihuman leukaemic lysozyme serum was seeded with the micrococcus. Two discs, each containing neat tear fluid from normal volunteers, were placed on the seeded agarose and incubated at 37°C for 18 hours. Two discs, from the same volunteers, were also placed on seeded agarose which did not contain antilysozyme serum and were similarly incubated.

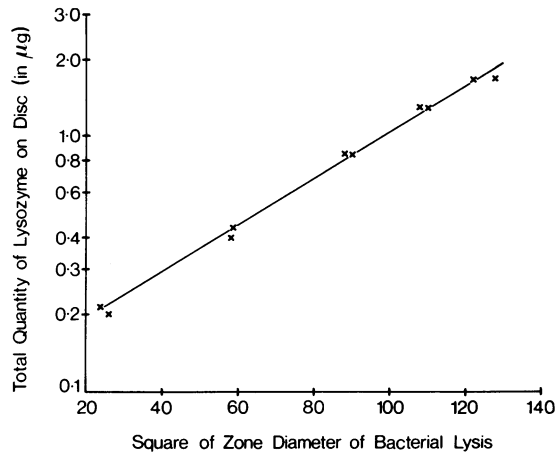

Fig. 3 *Calibration graph for plate assay.*

Results

THE ASSAY

The hen egg lysozyme (HEL) standard had an activity of 24 400 units per gram. This compared with an activity of 30 000 units per gram for our HEL standard acquired in 1975. By expressing all results in units of activity per μl, compensation was made for the 2 standards of different strengths, and results of these methods could be directly compared.

A standard graph was prepared for each plate assay plotting the logarithms of the total quantities (in micrograms) of lysozyme on the discs against the squares of the zone diameters of bacterial lysis (Fig. 3). The concentration of lysozyme in the tear eluate, and hence that in the original tear fluid, was calculated in units of activity per microlitre, and its relationship to the age of the patient is shown in Fig. 4. The limit of sensitivity for the method was 20 units of activity per μl tear fluid. The mean normal value decreased by 0·5 units of activity per μl tear fluid per year. Values for +/− 2 standard deviations (SD) of the means are shown together with the critical lower limit of the 'direct' method of assay which was 1·7 SDs below the mean at any age by this method; values below this limit for any age have only a 5% chance of normality, while values below the 2SD limit have only a 2·5% chance.

In order to make direct comparison between results of patients of different ages, adjustment was made by dividing each result, in units of activity per μl, by the value of the critical lower limit, given

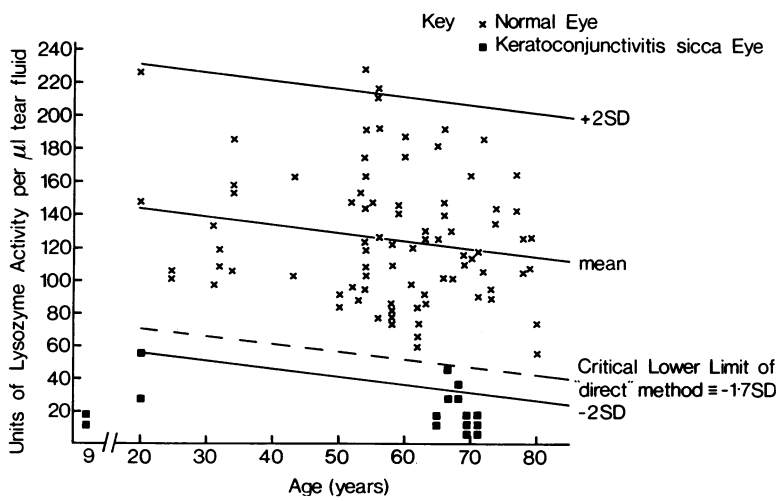

Fig. 4 *Lysozyme concentration by age in 42 normal patients and 8 patients with sicca.*

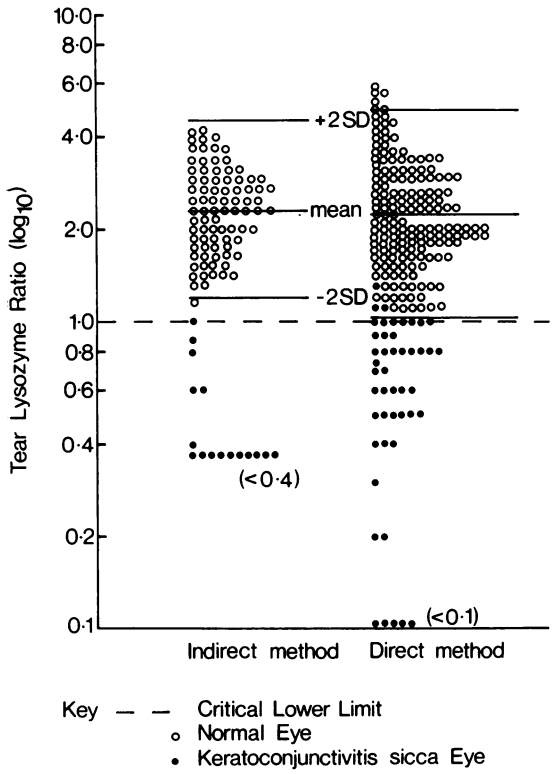

Fig. 5 *Distribution of tear lysozyme ratios.*

in Fig. 4, for that patient's age (tear lysozyme ratio (TLR)).

EVALUATION OF ASSAY

The distribution of the TLRs for the 'indirect' and 'direct' methods of assay is shown in Fig. 5, where means and SD limits for the normal range are shown, being different to those of Fig. 4 from which they have been derived; the TLRs are expressed on a logarithmic scale. There was no statistical difference between either method for the TLR values obtained from normal patients. At any age the critical lower limit is equivalent to a TLR of 1·0, so that values less than this will have only a 5% chance of normality. The lowest TLR value, and hence tear lysozyme concentration, that can be measured by the 'indirect' method is 0·4, while the lowest value for the 'direct' method is 0·1, because neat tear fluid was placed on to the agar plate resulting in larger zones of bacterial lysis.

For patients with keratoconjunctivitis sicca the TLR results by the 'indirect' method ranged from 1·0 to <0·4 and for the 'direct' method from 1·3 to <0·1. A TLR value of <0·4 is a satisfactory lower limit for this method, occurring in the patient with clinically established keratoconjunctivitis sicca and indicating that the lysozyme concentration is very low. This has diagnostic and pathological implications.

TEST FOR ANTIBACTERIAL SUBSTANCES

There was no lysis of the micrococcus around the 2 discs on the seeded agarose containing anti-lysozyme antiserum, though immune complex precipitates did occur under the disc, where lysis was also present. There were large zones of bacterial lysis around the 2 discs on the seeded agarose without antiserum. This showed that the tear lysozyme had been prevented from diffusing away from the disc and that there were no proteins present in the neat tear fluid which could inhibit growth of this micrococcus.

Discussion

There are various methods for lysozyme assay. We consider that the 'direct' method we previously described[1] added greatly to the accuracy of the test. Hypher[5] has shown that the method of Bonavida and Sapse,[6] whereby a 5 mm piece of a wetted Schirmer strip is cut off and used for the assay, is prone to inaccuracy because of irregular diffusion of tears along the strip. The disadvantage of our 'direct' method is the fact that discs have to be collected in the proximity of the laboratory and processed immediately. Processing can take up to 4 hours and often continues after normal laboratory hours. The 'indirect' method now described permits processing of discs at any time after collection and transportation of specimens to remote laboratories.

It has been pointed out[7] that there is a diurnal rhythm of tear lysozyme secretion, and we suggest that the discs are collected in the morning or afternoon to achieve maximum values.

The use of live rather than killed *Micrococcus luteus* has the advantage that a nutrient agar plate can be flooded when required and does not need to be prepared in advance. It is a suitable technique for routine use in a hospital laboratory, and sophisticated equipment is not required. The zone sizes obtained are clear to read. We have found no error from other antibacterial proteins known to be present in tear fluid, presumably because their concentration is too low or the *M. luteus* is not sensitive to them.

Lysozyme concentration in transported tear fluid has been studied before, when 1000 Schirmer test strips were posted to a laboratory.[8] The Schirmer strips had been wrapped in plastic film to preserve their moisture and eluates from them were tested by

filter paper electrophoresis. A study was made of the relative proportions of tear lysozyme, globulin, and albumin with semiquantitation of the tear lysozyme concentrations. However, the paper electrophoresis method is up to 300 times less sensitive than an agarose-micrococcus plate assay, but standard values were not quoted.[6] Rocket electroimmunoassay is more sensitive than paper electrophoresis but is not recommended for detecting low concentrations of lysozyme.[9]

The normal range of lysozyme concentration by the 'indirect' method has been found statistically similar to that using fresh neat tear fluid (direct method). This indicates that tear lysozyme remains stable when held in solution as an eluate. Abnormally low values have been found in patients with keratoconjunctivitis sicca, similar to those recorded by the 'direct' method, but a lower concentration can be detected by using neat tears. There is no possible loss of tear fluid from the disc as a deposit in the bottle with the 'indirect method' because phosphate-buffered saline is pipetted over the disc in the same bottle to form the eluate. In our earlier studies we found filter paper discs containing neat tear fluid, sealed in bottles, could not be held at +4°C, as they dried out by evaporation within the bottles. The lysozyme molecule is irreversibly damaged at the liquid-air interface when this occurs, and assay values fell considerably.

We envisage the development of enzyme linked immunoassay or radioimmunoassay for measuring the concentration of eluted lysozyme, using specific antiserum. It should be more sensitive, less time consuming, and would allow the processing of a larger number of specimens.

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