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A SIMPLE PROCEDURE FOR THE QUANTITATIVE EXTRACTION OF EXTRAVASATED DYE IN SKIN TISSUE

S. I. ANKIER and M. L. WHITESIDE

Department of Pharmacology, Allen & Hanburys Ltd., Ware, Herts U.K.

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Abstract—A rapid, simple and cheap procedure has been developed for the quantitative extraction of extravasated pontamine sky blue dye in skin tissue. The method is based on tissue digestion with NaOH, benzalkonium chloride and ethanol followed by colorimetric determination of the dye in solution. The procedure is applicable to a wide variety of experimental states involving the extravasation of dyes which are stable in aqueous alkali.

AS EARLY as 1867, Hoffman and v. Recklinghausen[1] noted that circulating colloidal dye accumulates at sites of injury and inflammation. Subsequently, the extravasation of colloidal dye into tissues was used as a qualitative measure of injurious stimuli.[2–6] Quantitative techniques were also developed based on visual scoring systems[7–16] but it has been found difficult to score the extent of extravasated dye if its appearance is mottled, patchy or irregular.[17] Less subjective quantitative methods have included direct comparisons with colour standards,[18,19] measurement of coloured areas[20,21] and colorimetric estimation of peritoneal exudate.[22] Tissue-bound dye has been extracted but such procedures are usually relatively complicated as tissues must be minced[23] or homogenised[24–27] before this is achieved. Tissue rupture has been avoided either by sample maceration with suramin for 14 days to elute the dye[28] or by drying specimens of skin before measuring extinction in a modified colorimeter.[29] Another colorimetric method for measuring the dye extravasated into tissues is that of Beach and Steinetz,[30] but a lengthy extraction, fractionation and separation is involved.

We now describe a rapid and simple method suitable for the routine extraction of protein-bound dye from skin tissue. The mouse pinna (auricula or flap of the external ear) is a convenient organ for this purpose as prior depilation is unnecessary. The method, which is applicable to any dye stable in aqueous alkali, depends on tissue digestion in the presence of NaOH, benzalkonium chloride and ethanol followed by colorimetric determination of the dye in solution. The procedure has been illustrated by the quantitative measurement of pontamine sky blue dye extravasated after the topical application of xylene[31] and the dextran pinnal anaphylactoid reaction.[32]

EXPERIMENTAL AND RESULTS

Reagents

The reagents used were NaOH pellets (May & Baker Ltd.), benzalkonium chloride (Berk Ltd.), ethanol (B.P. Chemicals Ltd.), pontamine sky blue 6BX dye (G. T.

Gurr Ltd.), xylene (Hopkin & Williams Ltd.) and 6% (w/v) dextran solution (Steriflex, Allen & Hanburys Ltd.). All reagents were dissolved in distilled water while solutions for intravenous injection were dissolved in isotonic saline.

Factors involved in the extraction

1. *Normality of NaOH.* 200 mg of mouse pinna, dissected across the concha and representing three pairs of whole pinnae, was incubated with 5 ml 0·5N, 1N, 4N or 8N NaOH at 40°. After incubation, the presence of a small lipid-like residue was eliminated by the addition of 90% (v/v) ethanol the optimal volume of which was 2 ml. Visual examination of the incubates revealed that complete digestion occurred at 10 hr in the presence of 1N, 2N, 4N, and 8N NaOH, but this process was not completed with 0·5N NaOH until 16 hr. It was decided to adopt 2N NaOH for the standard procedure since mixtures of 8N NaOH and pinnae were usually a yellow-orange colour.

2. *Temperature of incubation.* 200 mg of mouse pinna was incubated with 5 ml 2N NaOH at 20, 30, 40 or 50°, to which 2 ml 90% (v/v) ethanol was added after incubation. Visual examination of the incubates revealed that digestion was enhanced as the temperature was increased, complete digestion occurring at 40 and 50° after 10 hr of incubation.

3. *Recovery of added dye.* Accurately weighed portions of mouse pinna (30–250 mg) were placed in 5 ml 2N NaOH containing 25 μg/ml of pontamine sky blue dye. Since it was known that azovan blue dye (a structural analogue of pontamine sky blue dye) is strongly absorbed on to proteins and slightly absorbed on to glass at alkaline pH and that this is prevented by the cationic quaternary surfactant benzalkonium chloride,[33] 0·5 ml of either distilled water or 50% (w/v) benzalkonium chloride solution was added to each incubation mixture. After incubation overnight (approximately 18 hr) at 40°, 2 ml 90% (v/v) ethanol was added to each digest. In those digests without benzalkonium chloride, a blue precipitate was observed which increased in direct relation to the weight of pinna present and sedimented within 1 min. After allowing any precipitate to sediment, the extinction of each sample was read on a Unicam S.P. 1300 colorimeter fitted with a No. 626 Ilford Filter. When the weight of mouse pinna in the incubate exceeded 50 mg there was a significant loss ($P>0{\cdot}01$) of pontamine sky blue dye from the solution in the absence of benzalkonium chloride (Fig. 1).

The final procedure adopted as the standard method for extracting dye extravasated *in vivo* was to digest two or three pairs of mouse pinna in 5 ml 2N NaOH to which 0·5 ml 50% (w/v) benzalkonium chloride solution was added. After digestion overnight at 40° and the addition of 2 ml 90% (v/v) ethanol, extinction values were read colorimetrically on a Unicam S.P. 1300 colorimeter fitted with a No. 626 Ilford Filter (545–635 mμ). Using this method, added pontamine sky blue dye (25 μg/ml) was stable in solution for at least a week and the calculated recovery from digests was 96·1 per cent ($\pm$4·7).

Compliance with Beer's law

Although the absorption maxima for pontamine sky blue dye in distilled water is 620 mμ (measuring using an Unicam S.P. 800 Spectrophotometer) a shift to 590 mμ

occurs when the dye is dissolved in 2N NaOH and 50% (w/v) benzalkonium chloride (10 parts to 1). Nevertheless, Beer's Law is obeyed with final concentrations of pontamine sky blue dye between 0·5 μg/ml and 50 μg/ml irrespective of the presence of digested pinnae (Fig. 2).

Alkaline haematin as a possible interfering substance

NaOH reacts with haemaglobin to form a coloured complex, alkaline haematin.[34] Since active hyperaemia and stasis occur during the initial phase of inflammation, it was important to determine whether alkaline haematin interfered with the colorimetric estimation of extracted dye. However, using analysis of variance, the extinction of 200 mg of pinnae from mice treated with xylene (40 μl/ear) or dextran (500 mg/kg, intravenously) and control pinnae were not significantly different ($P>0·05$). Moreover, the addition of mouse whole blood in concentrations as high as 3·0 μg/ml of digest to normal pinnae did not alter the recovery of known amounts of added dye ($P > 0·05$).

QUANTITATIVE ESTIMATION OF DYE EXTRAVASATED *IN VIVO*

Intravenously injected pontamine sky blue dye (25, 50, 75, 100, 150 or 200 mg/kg) was extravasated in the mouse pinna after the topical application of xylene or the dextran pinnal anaphylactoid reaction. In all of these experiments, male mice (body weight 18–25 g) were used and the i.v. dose-volume was always 0·2 ml/20 g body weight.

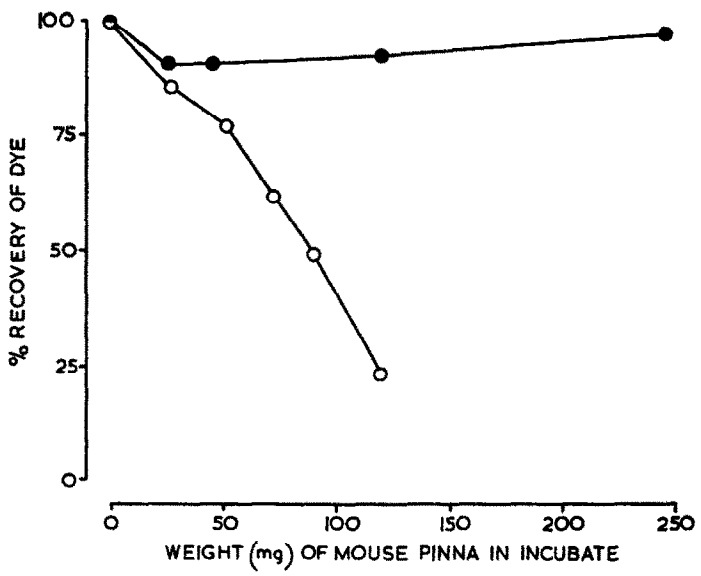

FIG. 1. Percent recovery of pontamine sky blue dye (25 μg/ml) from digests of mouse pinna (30–250 mg) with (●——●) or without (○——○) the addition of benzalkonium chloride. Each value is the mean of four replicates.

1. *The acute inflammation induced by the topical application of xylene*

Groups of mice (ICI strain) were injected i.v. with different concentrations of pontamine sky blue dye 30 min before xylene (40 μl/ear) was applied topically to the outer aspect of each pinna. The mice were killed 30 min later and the amount of dye extracted from their pinnae was determined colorimetrically (Fig. 3).

2. *The pinnal anaphylactoid reaction induced by the systemic injection of dextran*

There is a strain variation in the response of mice to systemically injected dextran. For example, dextran in an i.v. dose as high as 1800 mg/kg failed completely to

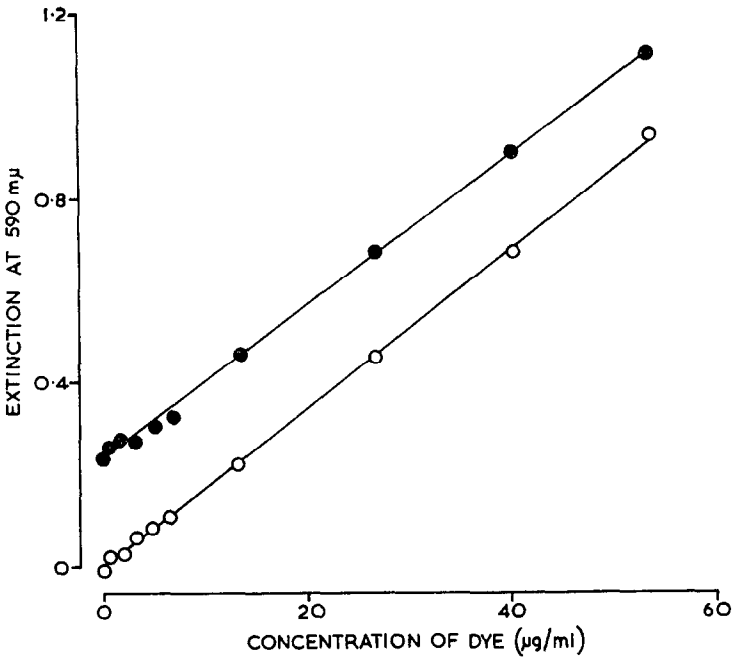

FIG. 2. A typical standard curve for pontamine sky blue dye in the presence (●——●) or absence (○——○) of 200 mg of mouse pinna digest.

elicit a pinnal anaphylactoid reaction in 134 ICI strain mice. However, out of 62 T_1O strain mice injected i.v. with dextran (500 mg/kg), only 16 failed to show a pinnal reaction. Accordingly, groups of T_1O strain mice were injected i.v. with dextran solution (500 mg/kg) in which different amounts of pontamine sky blue dye were dissolved. The mice were killed 2 hr later and the amount of dye extracted from the pinnae of those animals which had responded to the dextran was determined colorimetrically (Fig. 3).

After an inflammatory stimulus of standard intensity, the amount of pontamine sky blue dye extravasated was directly proportional to the amount injected for doses between 25–200 mg/kg.

DISCUSSION

In most biological work it is essential to quantify those parameters being studied. This is best performed using a reliable objective procedure so eliminating errors

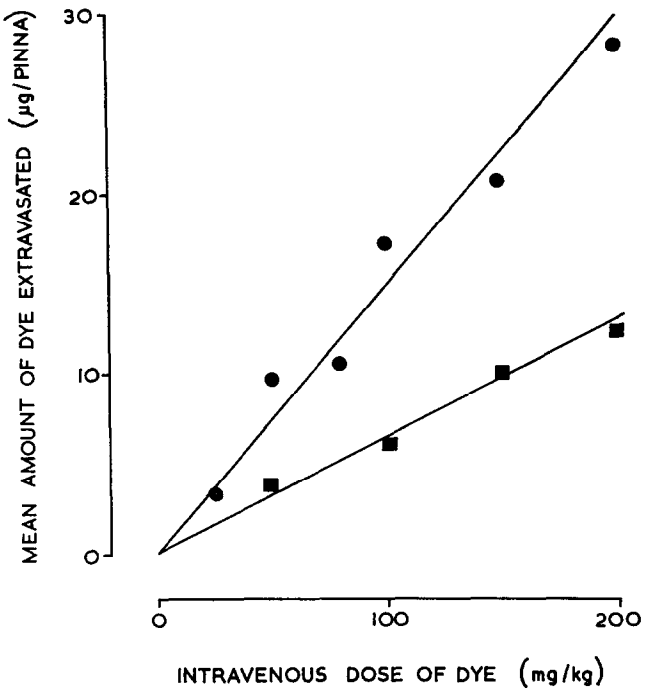

FIG. 3. Extravasation of different doses of pontamine sky blue dye after topically applied xylene (●——●) or the dextran pinnal anaphylactoid reaction (■——■).

arising from a subjective appraisal. Published procedures for objectively measuring the extent of extravasated colloidal dye in skin tissue are all relatively complicated. The procedure described in this paper, however, is not only rapid and cheap but little technical skill is required for its successful routine performance.

Two other diazo dyes, azovan blue and trypan blue, behave similarly to pontamine sky blue on extraction from the mouse pinna. Moreover, all of these dyes have been extracted quantitatively from the pinnae of the rat and guinea-pig as well as from the skin of the mouse, rat and guinea-pig. In these latter cases, however, depilation is necessary and the incubation time may have to be extended to 48 hr. The extraction procedure described should have many applications in quantitative studies where the extent of extravasated dye needs to be assessed objectively.

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