Physicochemical studies of indocyanine green (ICG): absorbance/concentration relationship, pH tolerance and assay precision in various solvents

Ó. G. Björnsson, R. Murphy and V. S. Chadwick

Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 OHS (England), 9 July 1981

Summary. Indocyanine green (ICG) obeyed the Beer-Lambert law within the concentration range 1.25 µg/ml–10.0 µg/ml in distilled water, methanol, dimethylformamide (DMF), 1.2-propanediol and aqueous buffers (pH 9.0), but only up to 7.5 µg/ml in human bile and 0.5% human albumin, and only to 5.0 µg/ml in human duodenal fluid. ICG was rapidly (< 1 h) decomposed to a colorless derivative at pH < 5 and > 11, but remained relatively stable for 48 h at pH 8–10. ICG is an indicator and a weak acid with a pKa of 3.27. In bile stabilized with 25% methanol, the precision of the method (CV) is 5% and the accuracy is 106–127%.

ICG (Cardiogreen®) is a water soluble, nontoxic tricarbocyanine dye of a mol.wt 775[1,2] (fig. 1). It was developed in the Kodak Research Laboratories by Heseltine and Brooker[1,2], and introduced into clinical research by Fox et al.[3] in 1956. It has since been used extensively for measurement of blood flow through organs in man and animals[4–9], and as a test substance for hepatobiliary excretion[4,5,9–16]. When administered i.v. ICG is almost entirely confined to the blood compartment because of molecular size and protein binding[2,3]. It is rapidly and exclusively extracted from the blood by the liver and excreted into bile chemically unchanged[4,5,9,17]. It has no enterohepatic circulation[14]. Although ICG is for this reason suitable for hepatobiliary studies, there are difficulties with its spectrophotometrical analysis, because it is unstable on storage in aqueous solutions[2,18–21], when a progressive loss of color is observed. This instability of ICG is known to be affected by various factors, such as light, dilution and the nature of the solvent[2,20–22].

In view of the problems of measuring ICG, we have studied the relationship between spectrophotometric absorbance and concentration of ICG in various biological fluids and organic solvents and the precision and accuracy of measuring ICG in biological fluids. The effect of changes in pH on ICG measurement have also been studied, since the pH of intestinal fluid may vary considerably.

Materials and methods. Human albumin (Sigma A2386) was purchased from Sigma London and ICG from Hynson, Westcott and Dunning Inc., Baltimore, Md 21201, USA. All other reagents were from Merck or British Drug Houses. For determination of absorbance/concentration relationship of ICG in various solvents, ICG was dissolved in distilled water and diluted to the final concentration of 10.0 µg/ml (1.29×10^{-2} mmoles/l), 7.5 µg/ml (9.68×10^{-3} mmoles/l), 5.0 µg/ml (6.45×10^{-3} mmoles/l), 2.5 µg/ml (3.23×10^{-3} mmoles/l) and 1.25 µg/ml (1.61×10^{-3} mmoles/l) in methanol, 1.2-propanediol. DMF, single distilled water, human bile (bile salt concentration 12.0 mmoles/, 8.0 mmoles/l, 6.0 mmoles/l, 4.0 mmoles/l and 2.0 mmoles/l, respectively), 0.5% human albumin, boric acid-sodium borate buffer (pH 9.0), and human duodenal fluid (fasting, bilirubin and bile salts free). Immediately after preparation (< 1 h), λ_{max} of ICG was determined in these solutions in a double beam spectrophotometer (Pye Unicam SP800), against appropriate blanks and the values plotted on a graph as a function of concentration. The following values of λ_{max} for ICG were observed in the various solvents: methanol 780 nm; 1.2-propanediol 785 nm; DMF 785 nm; single distilled water 775 nm; human bile 805 nm; 0.5% human albumin 795 nm; boric acid-sodium borate buffer 795 nm, and human duodenal fluid 775 nm.

The pH tolerance of ICG was tested by diluting ICG to a final concentration of 10 µg/ml (1.29×10^{-2} mmoles/l) in appropriate buffers of pH range from minus 0.5 to plus 13.0[23]. The samples were stored in room light (300 lux) and at room temperature (20 °C), and the absorbance of ICG in these buffers was read at λ_{max} (775 nm) at < 1, 18 and 48 h after preparation and the absorbance plotted as a function of pH. The dissociation constant of ICG (pKa) was determined spectrophotometrically at concentration 10 µg/ml (1.29×10^{-2} mmoles/l) by the method of Flexser, Hammett and Dingwall[24],

$$pKa = pHe - \log\frac{de - dbH^+}{db - de}$$

where db = maximum absorbance of the free base (λ_{max} at pH 7.4); dbH^+ = absorbance of the conjugate acid (λ_{max} at pH 1.0); and de = absorbance of the equilibrium mixture (λ_{max} at pH_e 3.2). The precision and accuracy of measuring ICG was determined at concentration 10.0 µg/ml (1.29×10^{-2} mmoles/l), 5.0 µg/ml (6.45×10^{-3} mmoles/l) and 2.5 µg/ml (3.23×10^{-3} mmoles/l) in n-saline and bile

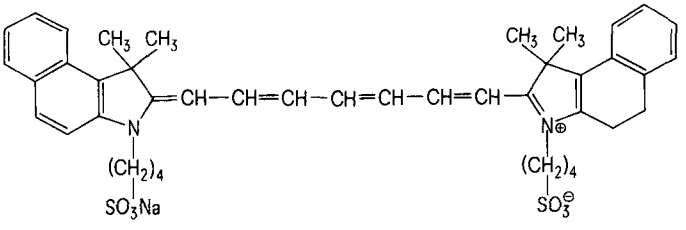

Figure 1. The structural formula of indocyanine green: anhydro-3,3,3′,3′-tetramethyl-1,1′-(sulfobutyl)-4,5,,4′,5′-dibenzoindotricarbocyanine hydroxide sodium salt. Mol.wt. 775.

Within-assay-precision (n = 20) and accuracy of measuring ICG in normal saline and human bile with or without 25% methanol

	0.9% NaCl			0.9% NaCl + 25% methanol			Human bile			Human bile + 25% methanol		
ICG µg/ml	2.5	5.0	10.0	2.5	5.0	10.0	2.5	5.0	10.0	2.5	5.0	10.0
$\bar{x}$	2.8	5.7	10.7	3.1	6.0	9.9	2.0	5.1	8.3	2.9	5.8	11.8
SEM	0.13	0.16	0.13	0.05	0.17	0.18	0.06	0.07	0.03	0.02	0.03	0.13
CV	19.90	12.30	5.50	7.02	12.67	8.17	14.50	6.00	1.80	2.48	2.65	4.99
Range	1.7–3.5	3.9–6.4	9.4–11.6	2.8–3.7	5.4–7.5	8.5–11.8	1.6–2.8	4.4–5.8	8.2–8.6	2.8–3.2	5.3–6.0	10.8–12.7

with and without 25% methanol as a stabilizing factor. Appropriate calibration curves and blanks were prepared for each sample, the blanks being prepared by decolourization of ICG by mixing 12N HCl, distilled water and the sample together in the proportions 1:1:2, which gave the final pH < 1.0.

Results and discussion. In the present study we have found a linear relationship over the test concentration range of ICG (1.25 to 5-10 µg/ml) in organic solvents (fig.2,A). In biological fluids, however, linearity was found only up to 7.5 µg/ml for 0.5% human albumin and bile, and up to 5 µg/ml for duodenal fluid (fig.2,B). Figure 3 shows the absorbance of ICG plotted as a function of pH (range minus 0.5 to plus 13.0) < 1, 18 and 48 h after preparation. In fresh preparations there was little change in absorbance at pH 5-11. Above pH 11 and below pH 5 there was a sudden fall in absorbance and at pH < 0.1 the absorbance of ICG had become negligible. This was used to correct for interference of bile pigments when ICG was measured in bile, by using decolorized samples in strong acids as blanks[15,16]. The effects of pH on the absorbance of ICG had previously been studied by Fox and Wood[2] and by Jones Owen[25], who found no changes in absorbance of ICG in water or plasma at pH 6.0-8.0. ICG is a weak acid with pKa 3.27. and in fact it is an indicator. Its light brown color in acidic solutions can be restored at least partially to green by changing pH to neutral. The table shows the within-assay-precision (n = 20) and accuracy of measuring ICG in n-saline or bile with or without methanol. Precision (CV) was best in bile with methanol and usually better at high concentrations of ICG than low. The reason for the low recovery of ICG at high concentration of bile alone is probably due to the deviation of ICG from the Beer-Lambert law at high bile salts concentration[26].

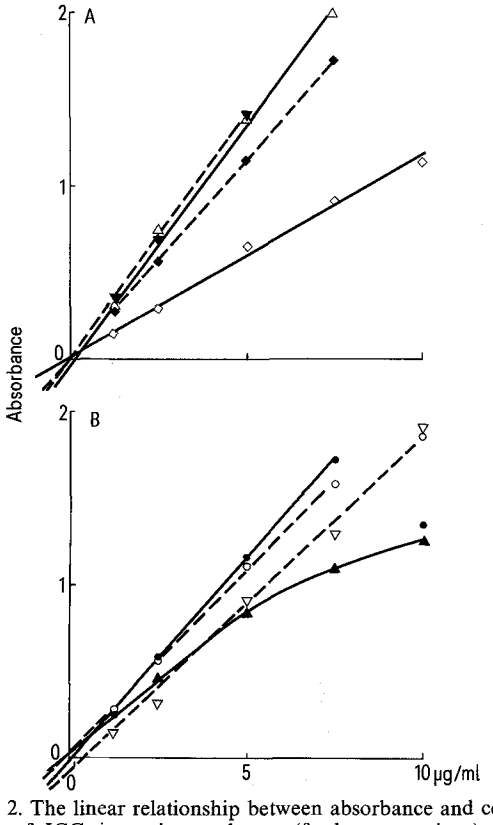

Figure 2. The linear relationship between absorbance and concentration of ICG in various solvents (fresh preparations). *A* ▼, methanol; △, 1.2-propanediol, ◆, dimethylformamide; ◇, distilled water. *B* ●, human bile; ○, 0.5% human albumin; ▽, 0.025M $Na_2B_4O_7$/0.1M H_3BO_3 buffer (pH 9.0); ▲, human duodenal fluid free of bile (fasting).

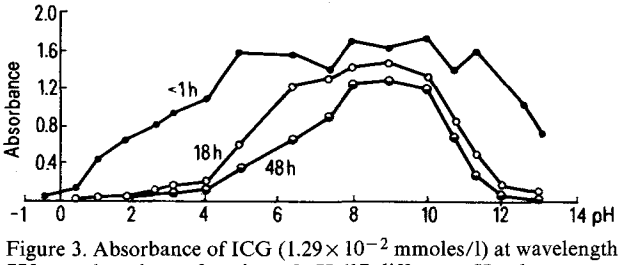

Figure 3. Absorbance of ICG (1.29×10^{-2} mmoles/l) at wavelength 775 nm plotted as a function of pH (17 different pH-values, range minus 0.5 to plus 13.0), < 1, 18 and 48 h after preparation.

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