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Physicochemical Studies on Indocyanine Green: Molar Lineic Absorbance, pH Tolerance, Activation Energy and Rate of Decay in Various Solvents

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Summary: Physicochemical studies were carried out on the tricarbocyanine dye indocyanine green in biological fluids and organic solvents. The molar lineic absorbance ε of the compound was highest in organic solvents (methanol, 1.2-propanediol, dimethylformamide) and bile, but lowest in water and duodenal fluid. Indocyanine green remained stable in methanol and bile ($t_{1/2} > 1$ year) but was rapidly decomposed to a colourless derivative in duodenal fluid and distilled water ($t_{1/2}$ 3.6 days and 1.4 days, respectively). It was thermostable (120 °C) in methanol and 1.2-propanediol but thermolabile in water and dimethylformamide where the activation energy for the decomposition reaction was low. At ambient temperature (20 °C) indocyanine green was particularly labile at pH < 5 and pH > 11. The rate of decay of indocyanine green in various solvents indicated that the rate limiting step in the decay process was either a first or zero order reaction.

Physikochemische Untersuchungen an Indocyaningrün:
Molare lineare Absorbanz, pH-Toleranz, Aktivierungsenergie und Zerfallsgeschwindigkeit in verschiedenen Lösungsmitteln

Zusammenfassung: An dem Tricarbocyaninfarbstoff Indocyaningrün wurden physikochemische Untersuchungen in biologischen Flüssigkeiten und organischen Lösungsmitteln durchgeführt. Die molare lineare Absorbanz der Verbindung war am höchsten in organischen Lösungsmitteln (Methanol, 1,2-Propandiol, Dimethylformamid) und Galle, am geringsten in Wasser und Duodenalsaft. Indocyaningrün war in Methanol und Galle stabil ($t_{1/2} > 1$ Jahr), wurde jedoch in Duodenalsaft und destilliertem Wasser sehr schnell zu einem farblosen Abkömmling zersetzt ($t_{1/2}$ 3,6 bzw. 1,4 Tage). Es war thermostabil (120 °C) in Methanol und 1,2-Propandiol, jedoch thermolabil in Wasser und Dimethylformamid, in denen die Aktivierungsenergie für die Zersetzungsreaktion niedrig war. Bei Umgebungstemperatur (20 °C) war der Farbstoff besonders labil bei pH < 5 und pH > 11. Die Zerfallsgeschwindigkeit von Indocyaningrün in verschiedenen Lösungsmitteln zeigte, daß der Zerfallsprozeß eine Reaktionskinetik erster oder nullter Ordnung folgt.

Introduction

Like most cyanine-dye chromogens indocyanine green (fig. 1) is sensitive to light and unstable on storage in aqueous solutions (1–5). Although there is some information available on this instability in diluted aqueous solutions and during exposure to light (1, 4–8), this has not been studied in detail in other solvents and little is known of the effects of temperature on indocyanine green (1, 4), and

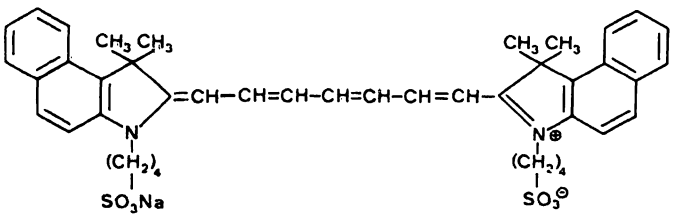

Fig. 1. The structural formula of indocyanine green: anhydro-3, 3, 3', 3'-tetramethyl-1, 1'-(sulfobutyl)-4, 5, 4', 5'-dibenzoindotricarbocyanine hydroxide sodium salt. M_r = 775.

Tab. 1. Physicochemical characteristics of indocyanine green in various solvents at constant pH.

	pH	λmax* (nm)	ε ($m^2 mol^{-1}$)	Tinctorial strength**	k***	$t_{1/2}$ (days)	Ea ($kJ mol^{-1}$)
Human duodenal fluid	6.3	775	11.8×10^3	1.68	1.3×10^{-2}	3.6	–††
Single distilled water	6.3	775	15.5×10^3	1.00	2.3×10^{-2}	1.4	26.9
Human albumin	6.1	795	17.1×10^3	1.86	1.3×10^{-4}†	81.0	–††
Dimethylformamide	11.6	785	21.7×10^3	2.12	1.8×10^{-3}	49.0	43.5
Methanol	7.3	780	23.4×10^3	2.43	5.6×10^{-4}	>1 year	15.6
1.2-Propanediol	8.1	785	25.1×10^3	2.30	2.1×10^{-4}†	90.0	17.3
Human bile	7.4	805	18.5×10^3	1.98	3.5×10^{-3}	>1 year	–††

* Measured in fresh solutions (<15 min after preparation).
** Calculated as the area under the absorption curves relative to that in distilled water as a solvent.
*** The unit of k for zero order reaction is mol $dm^{-3} h^{-1}$, and the unit of k for first order reaction is s^{-1}. Concentration of indocyanine green 6.45 µmol.
† Calculations were based on the period 7–30 days.
†† Not carried out in biological fluids.

changes in pH (1, 9). Because indocyanine green is used as a marker to monitor hepato-biliary excretion into the duodenum (10–12), where pH and composition of the luminal fluid is variable, we have in the present work studied the effects of storage and changes in pH on the spectro-photometric absorbance of indocyanine green in various biological fluids. The effects of high temperature on the rate of decay of indocyanine green in several organic solvents were also studied. The organic solvents and temperature were chosen as they might be of value in radiolabelling indocyanine green (13, 14).

Materials and Methods

Reagents used in this work were from British Drug Houses, Poole, England, Sigma London (albumin) and Hynson, Westcott and Dunning Inc., Baltimore, Ltd., 21201, U.S.A. (indocyanine green). Indocyanine green was dissolved in manufacturer's solvent and diluted to the final concentration of 6.45 µmol/l in methanol, 1.2-propanediol, dimethylformamide, single distilled water, human bile (bilirubin concentration 6.2 mmol/l; total bile salt concentration 12.0 mmol/l), human duodenal fluid (fasting, bilirubin free), and 72.5 µmol/l human albumin, respectively. The absorption spectrum of each solution was scanned against the pure solvent <1 h, 12 h, 48 h, 7 days, 1 month, 3 months, 6 months and 1 year after the preparation, using a Pye Unicam SP 800 double beam spectrophotometer. Solutions were kept in transparent sealed glass tubes in electric light (300 lux, corresponding to 0.44 Wm^{-2} at its maximum at 550 nm) and at temperature 18–20 °C during the period of storage.Molar lineic absorbance (ε) (15), tinctorial strength (the integrated absorption intensity) (16), half-lives ($t_{1/2}$) and rate constants (k) were calculated for indocyanine green in each solvent. Indocyanine green was diluted to the final concentration of 6.45 µmol/l in single distilled water, dimethylformamide, methanol and 1.2-propanediol. Aliquots were heated in sealed tubes to 120 °C for 2 h in an autoclave, and activation energy for the decay in each solvent calculated, based on absorption spectra before and after heating (14). Indocyanine green was dissolved and diluted to the final concentration of 12 µmol/l in appropriate buffers at pH 1.6, 5.0, 6.4, 9.0 and 12.6 respectively. The absorbance of each solution was scanned against the pure solvent over the wavelength range 600 nm to 1000 nm <1 h, 8 h, 24 h and 4 days after the preparation.

Results and Discussion

Physicochemical characteristics of indocyanine green in various solvents are shown in table 1. At constant pH the λmax (the wavelength of the absorption maximum) varied from being lowest in duodenal fluid and distilled water (775 nm) to being highest in human bile (805 nm). On the contrary there was no change in λmax in aqueous buffers at different pH (775 nm) as shown in table 2. The molar absorbance of indocyanine green had its minimum value in the duodenal fluid (tab. 1). This is in agreement with previous observations which show that electrolytes decrease the molar lineic absorbance (1). The molar lineic absorbance coefficient was highest in organic solvents.

Table 1 does also show that the tinctorial strength of indocyanine green was not constant, but varied from one solvent to another. This suggests that indocyanine green reacted with the solvents (aggregation effect) (16).

Tab. 2. Physicochemical characteristics of indocyanine green in buffers at various pH's.

pH	λ_{max}* (nm)	ε ($m^2 mol^{-1}$)	k**	$t_{1/2}$ (hours)
12.6	775	7.60×10^3	8.42×10^{-2}	2.0
9.0	775	12.6×10^3	1.31×10^{-2}	>24.0
6.4	775	13.2×10^3	6.59×10^{-2}	12.8
5.0	775	11.6×10^3	4.66×10^{-2}	3.2
3.2	775	10.7×10^3	9.84×10^{-2}	1.2
1.6	775	5.89×10^3	1.23×10^{-1}	2.0

* Measured in fresh solutions (<15 min after preparation).
** Based on the decay during the first 24 h after preparation. For units of k, see legend to table 1. Conc. of indocyanine green 12.9 µmol/l.

Inspite of the same molecular strength the molar lineic absorbance varied with changes in pH, being lowest in strongly alkaline and acidic solutions and highest in weakly alkaline solutions (pH 9).

The instability of indocyanine green on storage in diluted aqueous solutions was confirmed in this study. A progressive fall in absorbance at λmax was observed in all solutions (figs. 2–3), the absorption curves becoming broad and flattened on storage and the shoulder observed at wavelength 700 nm to 750 nm disappeared. The $t_{1/2}$ for decay of indocyanine green (6.45 μmol/l) in distilled water was 1.4 days, which is comparable with published figures (4).

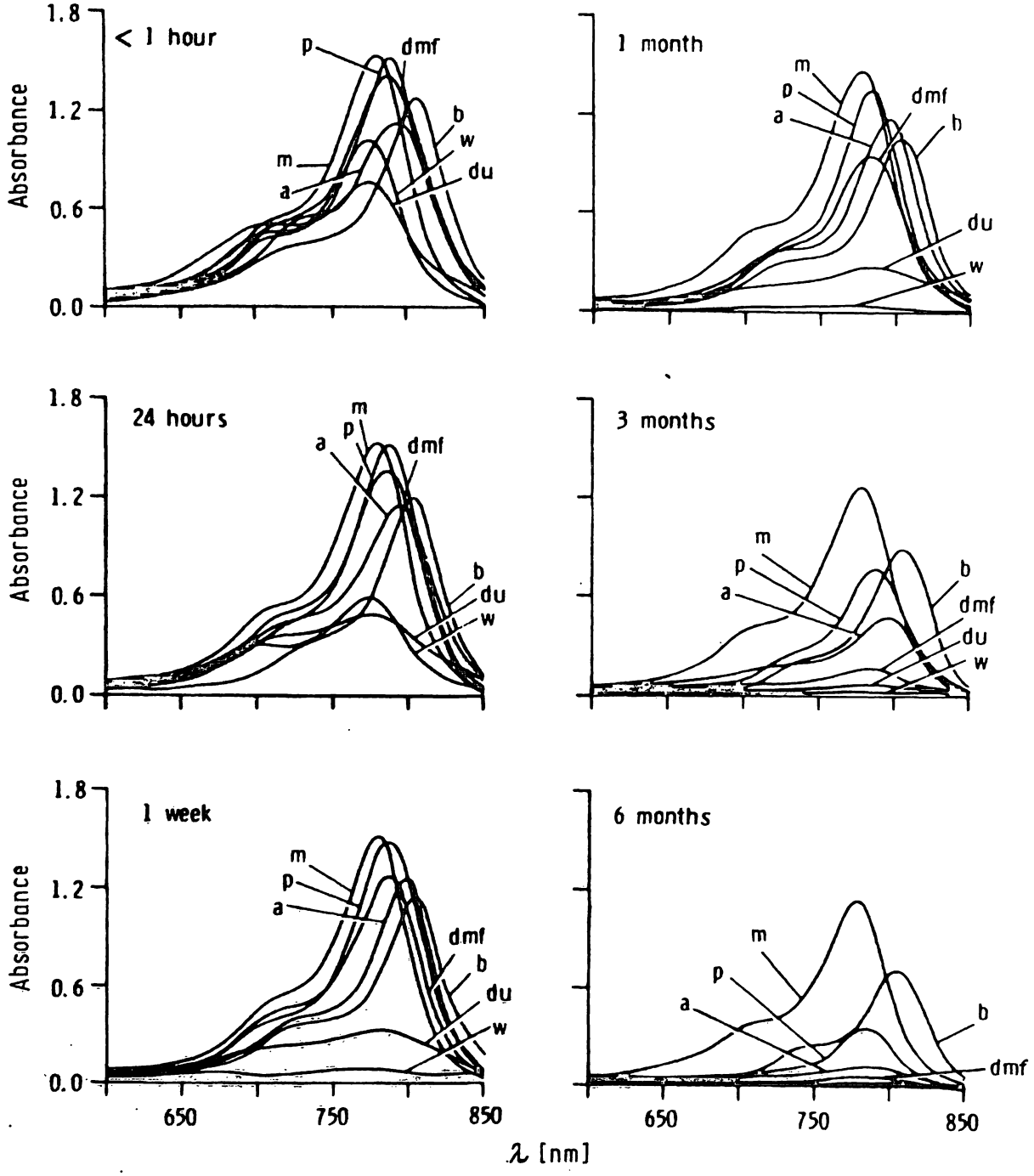

Fig. 2. Absorbance of indocyanine green (6.45 μmol/l) during 6 months of storage in various solvents drawn as a function of wavelength (λ) (600–850 nm);
a = 72.5 μmol/l human albumin;
m = methanol;
p = 1.2-propanediol;
dmf = dimethylformamide;
b = human bile;
w = distilled water;
du = human duodenal fluid free of bile (fasting).

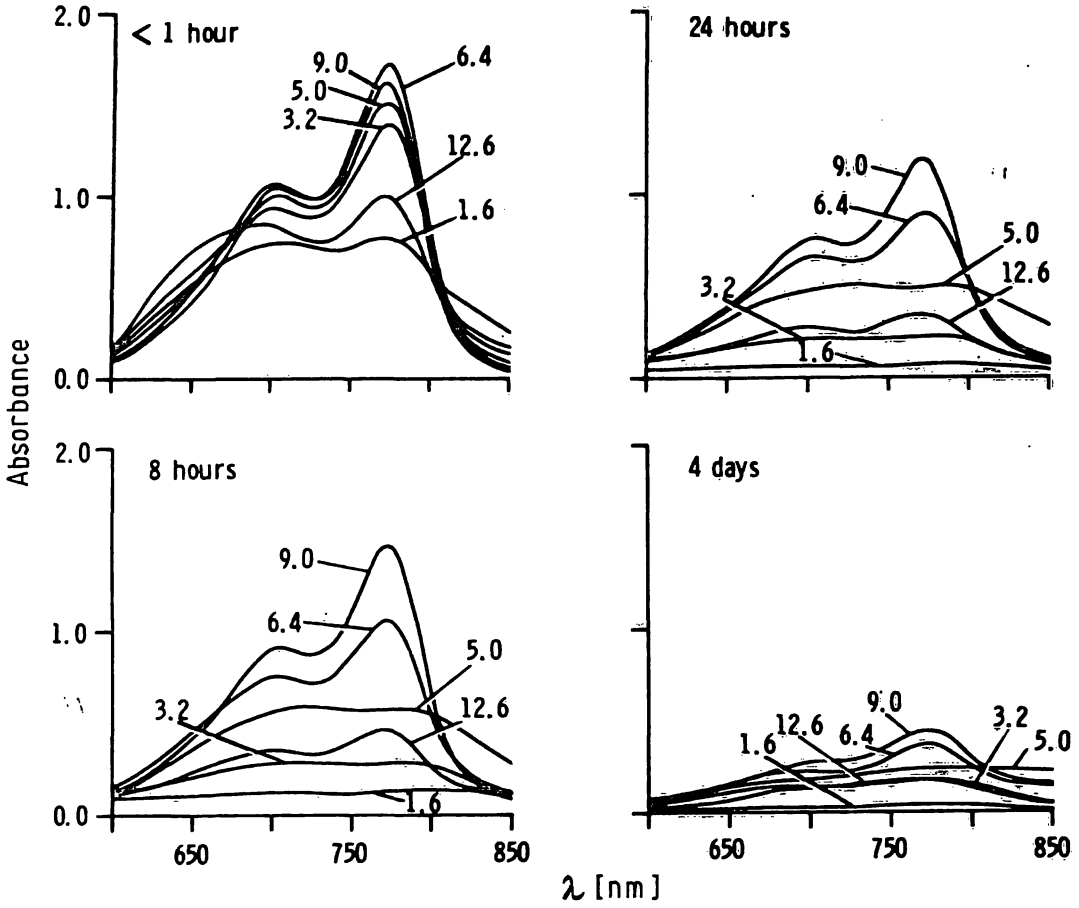

Fig. 3. Absorbance of indocyanine green (12.9 µmol/l) at pH 12.6, 9.0, 6.4, 5.0, 3.2 and 1.6, drawn as a function of wavelength (λ) (600–850 nm) <1 h, 8 h, 24 h and 4 days after preparation.

In duodenal fluid the $t_{1/2}$ was estimated as being 3.6 days, which was greater than expected, since electrolytes have been found to promote indocyanine green degradation (4, 5); the protein content of the duodenal fluid may, however, have opposed the ionic effect (4). Indocyanine green was relatively stable in dimethylformamide, human albumin and 1.2-propanediol, but most stable in methanol and bile ($t_{1/2}$ > 1 year). The stability in bile was found not to be due to protein, as there was no change in stability of indocyanine green in protein precipitated bile. The decay of indocyanine green was exponential in biological fluids (apart from human albumin), indicating that the decay was of first order reaction. In human albumin an increase in absorbance was observed during the first 7 days of storage (aggregation), after which indocyanine green decayed in a linear fashion, similar to that observed in organic solvents (zero order reaction). In aqueous buffers at various pH, $t_{1/2}$ varied from 1 to 2 h in strongly acidic (pH 1.6 to 3.2) and alkaline (pH 12.6) solutions (tab. 2) to 24 h at pH 9.0. The effect of pH on the absorbance of indocyanine green may be explained by the fact that many cationic dyes react with water at high pH values to form initially a "carbinol" type base, which is colourless. This compound may either degrade further to colourless compounds, or it can regenerate the original cationic dye on acidification. Similarly, the more hypsochromic colour in acid may result from protonation which gives dicationic species.

Indocyanine green seems to be relatively stable at the pH range which is expected in the intestinal lumen (pH 6.0–8.0). The decay curves in aqueous buffers were exponential.

It was possible to calculate the decay rate constant (k) for indocyanine green in various solutions and this correlated with $t_{1/2}$. Figure 4 shows that the decomposition of indocyanine green when heated at 120 °C for 2 h was minimal in methanol and 1.2-propanediol, in contrast to almost a complete decay in dimethylformamide and distilled water. Based on this, the activation energy (Ea) of the decomposition reaction of indocyanine green in dimethylformamide and distilled water was calculated. It was found to be low, which suggested that there was a little energy barrier to be overcome for the decomposition reaction to occur in these solvents, and in fact this was lower than the thermal energy available at room temperature.

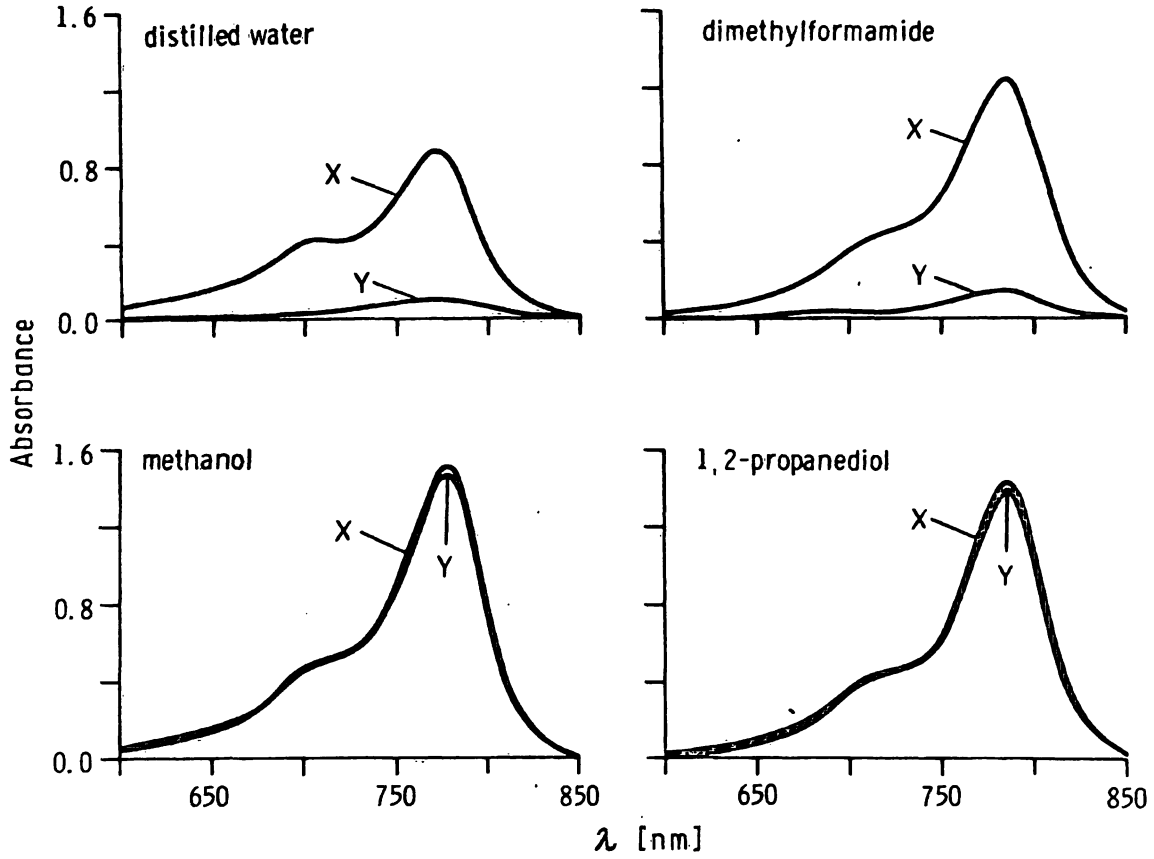

Fig. 4. Absorbance of indocyanine green (6.45 µmol/l) before (x) and after (y) heating (120 °C for 2 h) in distilled water, dimethylformamide, methanol and 1.2-propanediol, drawn as a function of wavelength (λ) (600–850 nm).

Activation energy measured at different temperatures is usually measured over several half-lives for the reaction; thus the stability of indocyanine green in methanol and propanediol (where $t_{1/2}$ is very low) precludes measurement of the activation energy in these solvents. It may be that in the more polar solvents (water and dimethylformamide) the relatively hydrophobic indocyanine green molecule adopts a tight, folded configuration which allows intramolecular re-arrangement of the molecule. In the more hydrophobic solvents (methanol and 1.2-propanediol) such a configuration may be disrupted by solvation and thus prevent the intramolecular re-arrangements that may be involved in the decay of indocyanine green. An intramolecular reaction mechanism seems likely from the kinetic data, which imply a unimolecular reaction governed by either zero or first order kinetics.

The results presented indicate that indocyanine green is not suitable for tritium labelling in aqueous or dimethylformamide media at high temperature or in acidic solutions even at ambient temperature (13).

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