in this table represents the amount released from a separate test sample. The values demonstrate the typical reproducibility obtained with this procedure.

This technique could be useful for testing a wide variety of topical formulations where direct contact with the receptor phase is desired. The screen size and configuration could easily be altered to accommodate different types of vehicles, release rates, and concentrations of drugs. This technique may be unsuitable where the drug or vehicle interacts with stainless steel. Such instances are expected to be rare, however, and normally the technique should allow investigators to routinely and uniformly prepare and test small samples with high surface area to weight ratios.

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Comparison of Chromatographic and Spectrophotometric Analysis of Indocyanine Green in Plasma Following Administration of Multiple Doses to Humans

Keyphrases □ High-pressure liquid chromatography—comparison with spectrophotometric analysis of indocyanine green in plasma following multiple dose administration, humans □ Spectrophotometry—comparison with high-pressure liquid chromatographic analysis of indocyanine green following multiple dose administration, humans □ Indocyanine green—comparison of spectrophotometric and high-pressure liquid chromatographic analysis following multiple dose administration, humans

To the Editor:

Following the administration of small doses (0.5 mg/kg), indocyanine green (I) is highly extracted from blood by the liver (1). Compound I has been used extensively, therefore, to evaluate hepatic function (2, 3) and to estimate hepatic blood flow in humans and laboratory animals (4, 5). Numerous reports have suggested that I is not metabolized in any species (4, 6, 7). Thus, simple spectrophotometric assays (*i.e.*, typically the determination of the absorbance at ~800 nm of plasma samples diluted with distilled water) have been used extensively to estimate the concentration of I in biological fluids. However, it has been reported recently that spectrophotometric and high-pressure liquid chromatographic (HPLC) assays yield radically different estimates of the concentration of I in plasma following the administration of a large dose (25.6 mg/kg) to the rabbit (8). Estimates of the plasma concentration of I ≥30 min postdose were found to be significantly lower using the HPLC assay (almost an order of magnitude lower at many time points). Thus, the total body clearance of I in the rabbit, calculated on the basis of plasma concentrations determined by HPLC, was much higher than that calculated from the spectrophotometric assay results. These investigators postulated that this discrepancy was due to a previously unidentified metabolite. If humans also metabolize I to a compound that interferes with the classical spectrophotometric methods, the clearance value of I based on these assays will not provide a reliable estimate of hepatic blood flow. Because of the potential implications of this assay discrepancy, we compared the spectrophotometric and HPLC assays for I using plasma samples from humans.

Two healthy male subjects (25 and 35 years of age) each received five intravenous bolus doses (0.5 mg/kg each) of I at ~1-hr intervals. Blood samples (5 ml) were collected into heparinized evacuated blood collection tubes[1] prior to and at 1, 3, 5, 7, 9, 11, and 15 min after the first, third, and fifth doses. Plasma was separated and stored at −20° until analyzed (within 36 hr). Previous studies have demonstrated the stability of I under these conditions (8), and preliminary work in our laboratory confirmed these findings.

After the addition of 1.0 μg of diazepam (internal standard) in 100 μl of methanol, proteins were precipitated with acetonitrile (1 ml plasma–1 ml acetonitrile) and the sample was centrifuged. The supernatant was then analyzed by spectrophotometric and HPLC methods. Samples were analyzed using a double beam spectrophotometer[2] equipped with a red-sensitive photomultiplier. The peak absorbance of I in the supernatant was found to occur at 786 nm, and this wavelength was chosen for analysis of all samples. The HPLC method used was that described recently (8) with the following modifications: Dual wavelength (254 and 650 nm)[3] monitoring was employed utilizing two detectors[4,5] in series. Absorbance at 650 nm was examined, since any degradation or metabolic products of I which contained an unaltered chromophore would be expected to absorb at a high wavelength similar to that of the parent compound. The high wavelength monitoring was achieved with a tungsten lamp in a variable wavelength monochromator[4]. A wavelength shorter than the maximum for absorbance by I was utilized in order to maintain an acceptable signal–noise ratio. Chromatography was performed on a reversed-phase column[6]. Of the various mobile phases studied previously (8), one composed of 0.05 *M* KH_2PO_4-Na_2HPO_4 (pH 6.0)–acetonitrile–methanol (50:47:3) was found to be most satisfactory. The peak height ratio was determined at both wavelengths relative to the diazepam peak height at 254 nm. Calibration curves were obtained in each subject's plasma for each analytical method.

[1] Vacutainer, Becton-Dickinson and Co., Rutherford, N.J.
[2] Model 25 Spectrophotometer, Beckman Instruments, Inc., Fullerton, Calif.
[3] Monitoring of column eluent was performed at 254 nm instead of 225 nm as in Ref. 8. This was advantageous since human plasma samples extracted as described above frequently contained a compound that had a retention time very similar to that of I and absorbed at 225 nm but not at 254 nm.
[4] Model 770, Schoeffel Instrument Corp., Westwood, N.J.
[5] Model 440, Waters Associates, Milford, Mass.
[6] μBondapak C_{18}, Waters Associates, Milford, Mass.

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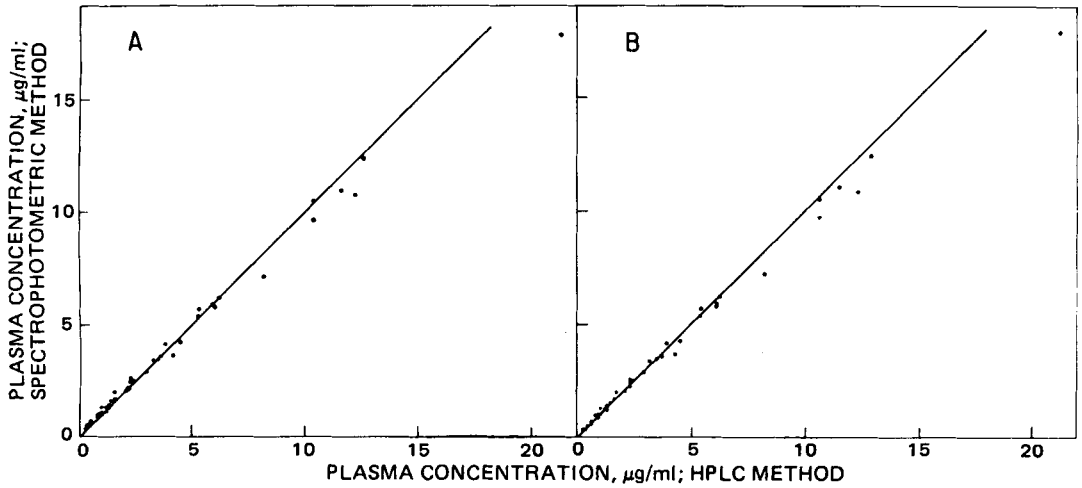

Figure 1—*Comparison of estimates of plasma indocyanine green concentration using spectrophotometric and HPLC procedures. Eluent was monitored at (A) 254 and (B) 650 nm.*

Figure 1A illustrates the relationship between estimates of the plasma concentration of I using the spectrophotometric assay and the HPLC assay (254 nm) which closely approximates the method that yielded the disparate rabbit data (8). As can be seen, a strong correlation exists between the results of the two procedures (r = 0.998; Spearman rank correlation). Similarly, a strong correlation was found between the results of the spectrophotometric method and the HPLC method with monitoring at 650 nm (r = 0.997; Fig. 1B) as well as between the two HPLC results (r = 0.999; data not shown). The close proximity of the data to the line through the origin with a slope of unity (Fig. 1) suggests that all of these assays provide comparable specificity. The single highly aberrant point (Fig. 1) was a sample drawn immediately after the first bolus; hence, the substantial difference between the two methods could not be attributed to accumulated metabolites. Indeed, the HPLC estimate was much higher than the spectrophotometric estimate.

The clearance of I (obtained by least-squares regression of the log plasma concentration *versus* time curve and the relationship: clearance = volume of distribution × elimination rate constant) estimated from concentrations determined by the spectrophotometric as well as HPLC assays at 254 and 650 nm was essentially identical (711 ± 144, 710 ± 145, and 695 ± 142 ml/min, respectively; n = 6; mean ± SD). No detectable concentration of I was present in plasma just prior to the third or fifth dose in either volunteer (lower limit of detection with HPLC method is 0.2 μg/ml). This suggests that accumulation of I does not occur following administration of small doses at 1-hr intervals. No extraneous chromatographic peaks (*i.e.*, peaks other than I and internal standard) appeared at any time points at either wavelength. However, this does not rule out the existence in humans of a metabolite similar to that suggested previously (8), since the metabolite which they proposed eluted in the solvent front, and in the present study this component of the eluent was not investigated. It is also possible that measurable quantities of such a metabolite are only formed when the capacity for biliary excretion is exceeded and, therefore, only seen following large doses or in selected species (*i.e.*, the rabbit) (9). However, the data presented in Fig. 1 suggest that the spectrophotometric assay utilized in this study and the HPLC procedures yield essentially identical estimates of the concentration of I in plasma following typical doses (0.5 mg/kg) in humans.

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