Journal of Chromatography, 417 (1987) 190-196
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3615

Note

Comparison of the spectrophotometric and high-performance liquid chromatographic analysis of Indocyanine Green in estimating systemic clearance in patients with chronic liver disease

T. O'REILLY*, P. MAC MATHÚNA, P.W.N. KEELING and J. FEELY

Department of Pharmacology and Therapeutics and Department of Clinical Medicine, Trinity College Medical School, St. James's Hospital, Dublin 8 (Ireland)

(First received September 22nd, 1986; revised manuscript received January 26th, 1987)

Indocyanine Green (ICG) is removed from the circulation specifically by the liver. ICG is a tricarbocyanine dye first developed for use in colour emulsion. Because of its characteristic absorption spectrum it has been used extensively as an indicator in dye dilution techniques for the determination of cardiac output [1, 2], hepatic blood flow [3, 4] and hepatic function [5, 6]. Following intravenous injection ICG is rapidly bound to plasma protein of which albumin is the principal carrier (95%). ICG is taken up from the plasma almost exclusively by the hepatic parenchymal cells and is secreted entirely in the bile. It is non-conjugated and there is no evidence of extrahepatic removal or of an enterohepatic circulation. After biliary excretion, the dye appears in the hepatic lymph independently of the bile suggesting that the biliary mucosa is sufficiently intact to prevent diffusion of the dye although allowing diffusion of bilirubin. These characteristics make ICG a helpful index of hepatic function.

ICG plasma concentrations have traditionally been determined by measurement of the spectrophotometric absorbance of unextracted plasma at approximately 800 nm. Recent studies using large doses of ICG in the rabbit [7] and preliminary data in healthy volunteers [8] suggest that the spectrophotometric method overestimates ICG. Whether this extends to the relevant group of patients is unknown. We, therefore, developed a modified high-performance liquid chromatographic (HPLC) method in patients with known liver disease to analyse ICG and compared it with the traditional spectrophotometric method of Caesar et al. [3].

0378-4347/87/$03.50 © 1987 Elsevier Science Publishers B.V.

EXPERIMENTAL

Chemicals and reagents

All chemicals (KH_2PO_4 and Na_2HPO_4) were analytical reagent-grade and obtained from BDH (Poole, U.K.). ICG was supplied by Hynson Westcott & Dunning (Baltimore, MD, U.S.A). Reagents (acetonitrile, methanol and dichloroethane) used were of HPLC grade and purchased from Fisons Scientific (Loughborough, U.K.). The water used was doubly glass-distilled.

Patients and sample analysis

Five patients with chronic liver disease were studied before and following administration of a number of drugs (nifedipine, propranolol and a combination of propranolol and glyceryl trinitrate). The drugs studied did not interfere with the analysis. In each of the five patients a sampling cannula was placed in a peripheral vein. ICG was reconstituted immediately before use in aqueous solvent to concentrations of 2.5 mg/ml for injection. ICG (0.25 mg/kg) was injected as a rapid intravenous bolus over 15 s into a peripheral vein of an arm opposite to the sampling cannula. At time 2, 4, 6, 8, 10, 12, 15 and 20 min after completion of the injection, blood was drawn into lithium heparin tubes from the opposite arm peripheral vein and the tubes were inverted gently. The drugs (10 mg nifedipine sublingually, 0.15 mg/kg propranolol and 500 μg glyceryl trinitrate) were given over a period of 10 min. The same quantity of ICG was injected as a rapid intravenous bolus and the sampling repeated. Plasma samples were obtained following centrifugation (800 *g*) for 15 min at 20°C. Analysis was carried out immediately after obtaining the plasma samples.

Waters Model 510 pump and injector, Waters Model 441 UV detector and a Shimadzu C-R3A Chromatopac integrator comprised the chromatographic apparatus. Chromatography was performed on a reversed-phase column (10 cm×8 mm I.D., 5-μm Bondapak C_{18}) using the radial compression separation system. The mobile phase was acetonitrile–methanol–0.05 *M* KH_2PO_4–Na_2HPO_4 buffer (pH 6.0) (47:3:50). The acetonitrile concentration is critical for successful resolution of ICG. The use of the methanol sharpens and reduces the retention time of the internal standard. With respect to the buffer the salt strength is more important than pH for successful retention of ICG.

A spectrophotometric scan of ICG in reagent-grade water showed a peak at 800 nm and another at 214 nm. To minimize interference from plasma constituents and to maximize detector performance, a non-optimal wavelength (225 nm) was used for detection purposes.

Spectrophotometric detection of ICG

Plasma (1 ml) was transferred to a microcuvette and recentrifuged again if necessary. The absorbance of the samples was read at 800 nm. The concentration of ICG was calculated pre- and post-drug using standard curves constructed from the control sample.

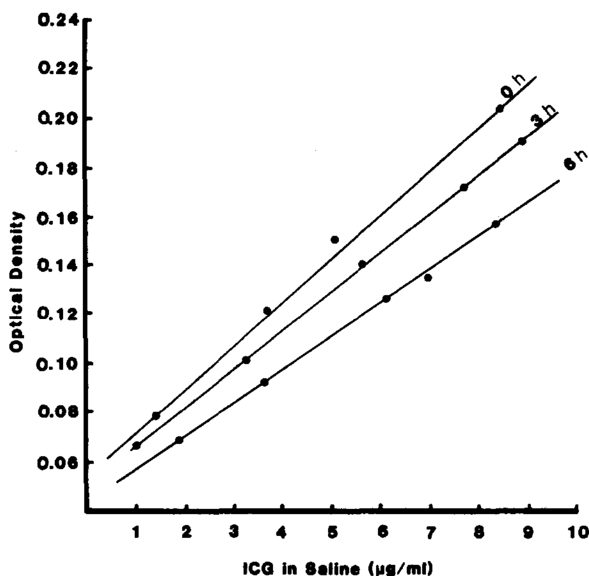

Fig. 1. Degradation of ICG.

Detection of ICG by HPLC

A fixed plasma volume (0.25 ml) was used for all samples. Acetonitrile was added in a 1.6:1 (v/v) ratio with plasma for precipitation of the proteins. Each sample was vortexed. Methanolic diazepam (5 μg in 50 μl) was added as the internal standard and the mixture was vortexed for 20 s. The samples were centrifuged at 800 *g* for 3 min and 25 μl were injected on to the column. After preparation the sample had to be injected immediately due to degradation of the ICG. The flow-rate used was 2.0 ml/min. The peak-height ratio was determined relative to diazepam at 214 nm.

RESULTS AND DISCUSSION

ICG degrades with time at room temperature (Fig. 1, $n=5$) so therefore analysis has to be carried out immediately after obtaining the plasma samples.

Calibration curves were obtained in each subject's plasma for each analytical method which were linear over the concentration range 0–10 μg/ml. The inter-assay coefficients of variation were 4.5% for 3 μg/ml samples ($n=6$) and 3% for 8 μg/ml samples ($n=4$) for the HPLC method and 2% for 2 μg/ml samples ($n=10$) and 1.8% for 8 μg/ml samples ($n=6$) for the spectrophotometric method. The overall recovery after extraction of the ICG was 89.6% for a 5 μg/ml sample of ICG. A typical HPLC profile is shown in Fig. 2.

Our modified HPLC method for ICG plasma concentrations yields a different plasma concentration–time profile from the spectrophotometric method (Fig. 3). Estimates of the plasma concentration of ICG were found to be significantly lower using the HPLC technique particularly over the interval 5–20 min following injection, being most marked in later samples when ICG concentration was low

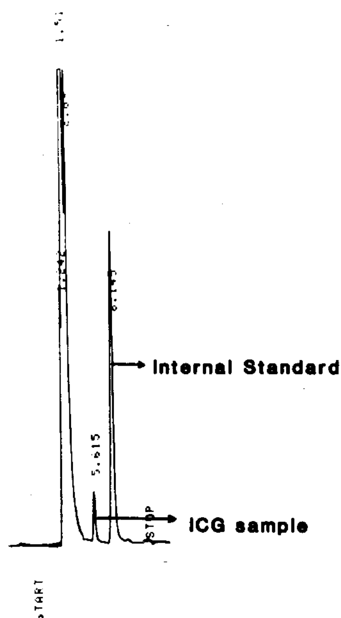

Fig. 2. HPLC profile of ICG (25 μl injection volume of a 1 μg/ml sample; 1 a.u.f.s.).

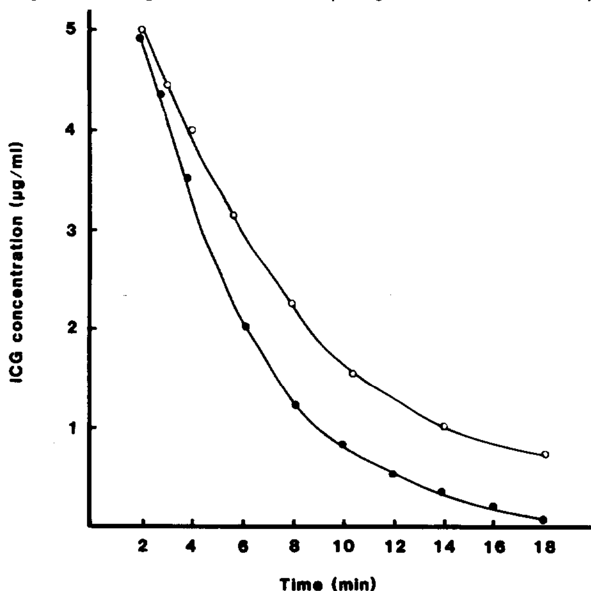

Fig. 3. ICG plasma concentration-time profiles (1 μg/ml sample of ICG) from the spectrophotometric (○) and HPLC (●) method.

(<1.5 μg/ml). Overall a strong positive correlation was found between plasma ICG concentrations analysed by the two methods ($r=0.96$; Spearman rank correlation, $n=35$, $P<0.01$) (Fig. 4).

The clearance of ICG was obtained by least-squares regression of the log plasma concentration versus time curve [9]. Statistical analysis was performed using a

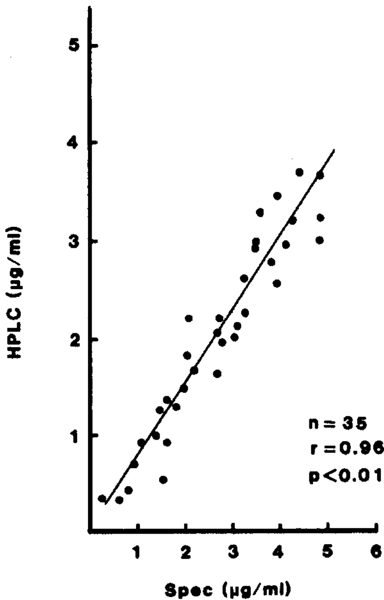

Fig. 4. Comparison of estimates of plasma ICG concentration using spectrophotometric (Spec) and HPLC procedures.

student's paired t-test. The total body clearance of ICG calculated on the basis of plasma concentrations determined by HPLC was much higher ($P < 0.05$) than that calculated from the spectrophotometric assay results.

The variables calculated were half-life (min), volume of distribution (l), systemic clearance plasma (ml/min) and percentage reduction in clearance due to therapy for both spectrophotometric and chromatographic methods. The mean and standard deviation of each of the kinetic variables are listed in Table I. There was no significant difference between the two analytical methods in the calculated volume of distribution or ICG elimination half-life. There was a small but

TABLE I

ICG PHARMACOKINETIC VARIABLES (MEAN ± S.D.) USING SPECTROPHOTOMETRIC AND HPLC METHODS

Variable	Spectrophotometry	HPLC
Half-life (min)		
Control	16.68 ± 14.51	17.13 ± 17.89
Drug	41.7 ± 60	29 ± 35
Volume of distribution (l)		
Control	3.23 ± 1.15	4.95 ± 2.71
Drug	2.64 ± 0.90	4.0 ± 2.50
Systemic clearance plasma (ml/min)		
Control	399 ± 256.20	530 ± 270
Drug	276 ± 212	338 ± 228
Reduction change in clearance (%)	40.6 ± 24.23	42.2 ± 14.9

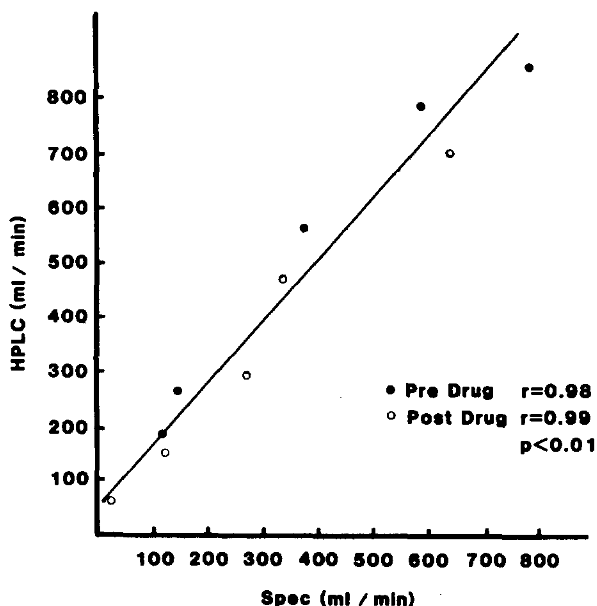

Fig. 5. Comparison of clearance between the two analytical methods.

significant difference between the two analytical methods in estimating systemic clearance, $P<0.01$ for pre-drug results and $P<0.05$ for post-drug results (Fig. 5). However, the percentage reduction change of drug between the two analytical methods in estimating clearance were not significantly different (40.6% for the spectrophotometric method and 42.2% for the HPLC method; Table I).

There are a number of possible explanations why the spectrophotometric method records higher ICG concentrations. The first suggestion by Donn et al. [8] and Rappaport and Thiessen [10] is that ICG undergoes some degradation or metabolism in the body prior to extraction. A metabolite may be released rapidly in the blood after ICG is taken up by the liver and absorbs light at 800 nm thus giving higher readings in the spectrophotometric method. The second possible explanation advanced by Christie et al. [11] is that ICG is not pure. They reported an additional peak eluting from the HPLC column prior to the parent dye suggesting a compound that is more polar than ICG.

CONCLUSION

It can be concluded that the HPLC provides a sensitive and specific method for ICG analysis. While the spectrophotometric method overestimates ICG in patient studies, the extent is small and primarily involves later samples and this traditional method reflects accurately drug-induced changes in systemic clearance.

ACKNOWLEDGEMENT

The authors wish to thank the Medical Research Council of Ireland for financial support.

REFERENCES

1 D.E. Miller, W.L. Gleason and H.D. McIntosh, J. Lab. Clin. Med., 59 (1962) 345.
2 B. Raffestin, J.P. Merillon, C. Masquet, P. Lorente and R. Gourgon, Coeur Med. Interne, 14 (1975) 305.
3 J. Caesar, S. Shaldon, L. Chiandussi, L. Guevara and S. Sherlock, J. Clin. Sci., 21 (1961) 43.
4 B.D. Wiegand, S.G. Ketterer and E. Rappaport, Am. J. Dig. Dis., 5 (1960) 427.
5 G.R. Clerrick, S.W. Stein, C.M. Leevy and C.S. Davidson, J. Clin Invest., 39 (1960) 592.
6 D.B. Hunton, J.L. Bollman and H.N. Hoffman, Gastroenterology, 39 (1960) 713.
7 J.J. Thiessen, P.L. Rappaport and J.G. Eppel, Can. J. Physiol. Pharmacol., 62 (1984) 1078.
8 K.H. Donn, J.R. Powell, J.F. Rogers and J.R. Plachetka, J. Clin. Pharmacol., 24 (1984) 360.
9 M. Rowland and T. Tozer, Clinical Pharmacokinetics, Lea & Febiger, Philadelphia, PA, 1980, pp. 65–75.
10 P.L. Rappaport and J.J. Thiessen, J. Pharm. Sci., 71 (1982) 157.
11 J.P. Christie, N.D.S. Bax, M.S. Lennard and G.T. Tucker, Br. J. Clin. Pharmacol., 21 (1986) 568.