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High-Pressure Liquid Chromatographic Analysis of Indocyanine Green

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Abstract □ The development of a specific high-pressure liquid chromatographic (HPLC) assay, having a quantitative detection limit of 0.4 μg/ml, is described for plasma indocyanine green. In a preliminary study, the HPLC method demonstrated that the traditional spectrophotometric procedure inaccurately quantitates the dye's degradation in aqueous medium since degradation products that absorb light at 770 nm are formed. In a further study, the spectrophotometric method yielded an erroneously low clearance of indocyanine green in the rabbit. In addition, the HPLC assay points to the possibility of metabolite formation of the dye *in vivo*.

Keyphrases □ Indocyanine green—high-pressure liquid chromatographic analysis □ High-pressure liquid chromatography—analysis of indocyanine green □ Dyes—indocyanine green, high-pressure liquid chromatographic analysis

Indocyanine green, an organic anion, has been used to determine cardiac output (1, 2), hepatic blood flow (3–5), and hepatic function (3–9). Analytical methods have been based on the dye's absorbance at 775–800 nm. These procedures employ either direct, continuous measurements of dye in circulating whole blood with a densitometer (1, 2, 10) or standard spectrophotometric analysis of harvested plasma following discrete blood sampling (3–9).

BACKGROUND

In vitro studies (10–12) determined that aqueous solutions of indocyanine green degrade quickly in a concentration-dependent fashion. However, in the presence of plasma or albumin, dye stability is much improved.

In vivo experiments demonstrated a saturable process for indocyanine green uptake by the liver (13, 14) and a biexponential decline in plasma levels for rats, rabbits, dogs, and humans (5, 6, 9, 15). Elimination of unchanged dye by the liver into bile has been thought to be the sole method for indocyanine green clearance. Evidence for this reported absence of metabolites was obtained from paper chromatographic analysis of plasma and bile samples (3, 5, 16, 17).

The reliability of this information depends on the perceived specificity of the spectrophotometric analysis method. Another procedure was needed to examine the existing methods critically. This report describes a high-pressure liquid chromatographic (HPLC) method for analysis of indocyanine green. Preliminary data are presented, highlighting discrepancies between the HPLC and spectrophotometric methods for the determination of *in vitro* dye stability and *in vivo* pharmacokinetics.

EXPERIMENTAL

HPLC Instrumentation—A high-pressure pump[1], a high-pressure injector[2], UV detector[3] (225 nm), and a recorder[4] comprised the chromatographic apparatus. Chromatography was performed on a reversed-phase column[5], using a reversed-phase precolumn[6] to extend column life. The mobile phase, at a flow of 2 ml/min, was composed of 47 parts of acetonitrile[7], 3 parts of methanol[7], and 50 parts 0.05 *M* KH_2PO_4–Na_2HPO_4 buffer[8] (pH 6). A spectrophotometric scan[9] of indocyanine green[10] in reagent grade water[11] showed a peak at 770 nm ($\epsilon = 1.43 \times 10^5$) and another at 210 nm ($\epsilon = 5.17 \times 10^4$). To minimize interference from plasma constituents and to maximize detector performance, a nonoptimal wavelength (225 nm) was used for detection purposes (ϵ ~60% of that at 210 nm).

Preparation of Standards—For the preparation of calibration curves, methanolic solutions of indocyanine green (20, 15, 10, 7, 4, 2, 1, 0.5, 0.2,

[1] Altex model 100.
[2] Valco Universal inlet HPLC valve.
[3] LDC Spectro Monitor I.
[4] Tracor Westronics MT.
[5] Hibar II, C_{18}, 10 μm, 250 × 4.6-mm i.d.
[6] Perisorb, C_{18}, 30–40 μm, 40 × 3.2-mm i.d.
[7] Glass distilled, Burdick & Jackson.
[8] Certified ACS, Fisher Scientific.
[9] Cary model 118, Varian.
[10] Cardio-Green, Hynson, Westcott and Dunning.
[11] Double distilled.

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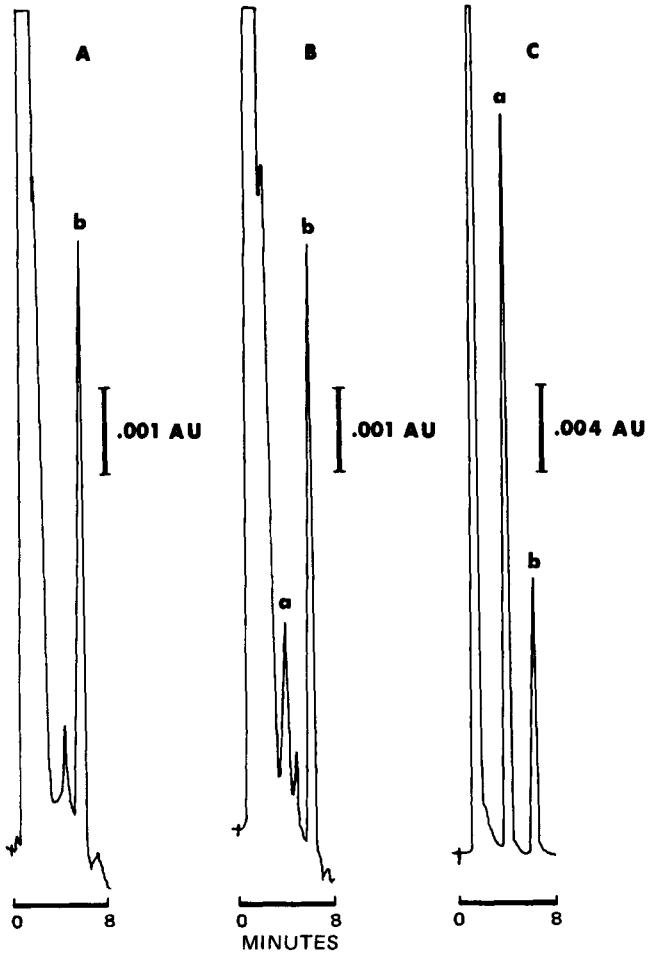

Figure 1—*HPLC chromatograms obtained for indocyanine green (a) and diazepam (b) using an RP-18 column (250 × 4.6-mm i.d.) and a mobile phase of 47% acetonitrile–3% methanol–0.05 M phosphate buffer (pH 6) at 2.0 ml/min. Detection was at 225 nm. Key: A, diazepam (0.15 μg/0.25 ml) in blank rabbit plasma; B, indocyanine green (0.1 μg/0.25 ml) and diazepam (0.15 μg/0.25 ml) in blank rabbit plasma; and C, indocyanine green (10 μg/0.25 ml) and diazepam (1.5 μg/0.25 ml) in blank rabbit plasma.*

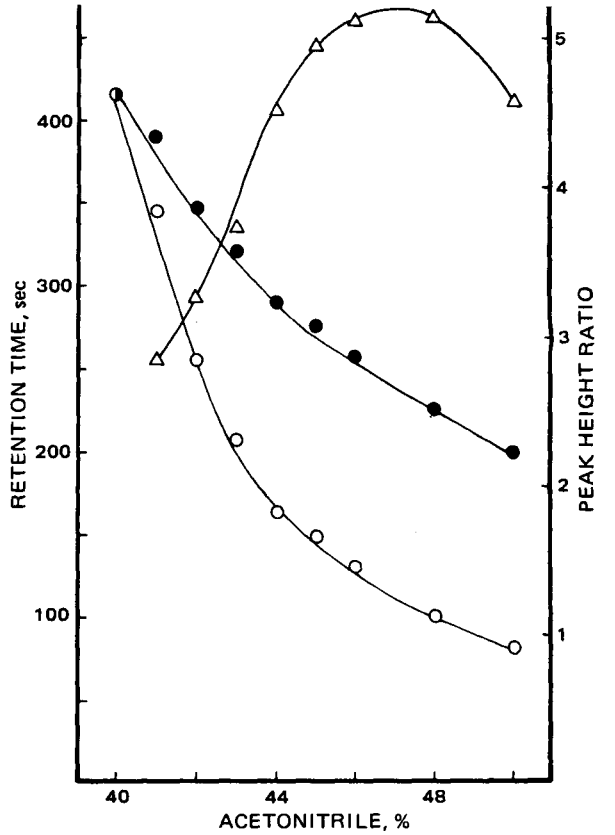

Figure 2—*Effect of acetonitrile concentration in the mobile phase on the retention times of indocyanine green (○) and diazepam (●) and on the indocyanine green/diazepam peak height ratio (△). The aqueous part of the mobile phase was composed of a 0.025 M phosphate buffer (pH 4). The mobile phase was run at 1.6 ml/min on an RP-18 column (250 × 3.2-mm i.d.).*

and 0.1 μg in 0.1 ml) were evaporated to dryness at 40° under a gentle dry nitrogen stream. After this step, standards could be stored in a desiccator under vacuum for 1 week with no detectable degradation. At the time of sample analysis, blank rabbbit plasma (0.25 ml) was added to each tube and allowed to stand for 30 min with intermittent vortexing. After this point, standards were processed in the same manner as *in vivo* samples.

HPLC Sample Preparation—A fixed plasma volume (0.25 ml) was used for all samples. Concentrated samples (>80 μg/ml) were diluted with blank rabbit plasma. Acetonitrile in a 1.6:1 (v/v) ratio with plasma was added in four aliquots (0.1 ml each for 0.25 ml of plasma), with vortexing after each addition. Methanolic diazepam[12] (5, 1.5, or 0.15 μg in 50 μl) was then added as the internal standard, and the mixture was vortexed for 20 sec. The sample was centrifuged(1700×*g*) for 2 min, and a volume of the supernate was injected onto the column. Due to recognized degradation of indocyanine green, the maximum allowable time between sample precipitation and column injection was 30 min.

Spectrophotometric Analysis—The plasma sample (0.25 ml) was diluted with 3.3 ml of reagent grade water. The absorbance of the solution was measured (800 nm) using similarly prepared blank rabbit plasma as the reference.

Stability of Aqueous Solutions—An indocyanine green solution (20 μg/ml in reagent grade water) was exposed to a constant amount of room light (420 lux) at 23°. The solution was sampled periodically to determine the amount of parent dye remaining.

For HPLC analysis, 100 μl of this solution and 100 μl of acetonitrile were added to a tube containing diazepam (0.26 μg). The mixture was vortexed and injected onto the column. Indocyanine green standards were prepared as already described. At the time of analysis, methanolic diazepam (0.26 μg in 0.05 ml) was added to the standards and evaporated. After reconstitution with 0.2 ml of 50% acetonitrile–reagent grade water, the standard was subjected to HPLC analysis.

Spectrophotometric analysis[13] (770 nm) was performed on the solution without a dilution step. The absorbance measurement of a freshly prepared dye solution served as the standard for comparison.

Stability of Plasma Solutions—Standard plasma samples containing 80, 40, 8, 4, or 0.8 μg of indocyanine green in 2.0 ml of fresh blank plasma were prepared as already described. The samples were kept at 6° in polyethylene tubes and 0.25 ml of each was sampled seven times over 10 weeks. A calibration curve, obtained from freshly prepared standards analyzed at the same time as the samples, was used for quantitation of dye degradation.

Indocyanine Green *In Vivo* Study—Indocyanine green was reconstituted with 5% dextrose–water to give a concentration of 8.8 mg/ml. A 25.6-mg/kg dose was administered intravenously[14] to a rabbit over 4 min. Thirteen blood samples (1.5 ml) were collected over 6 hr. Harvested plasma was kept at 6° until analysis later that day.

Calculations—The unknown indocyanine green concentrations in samples analyzed spectrophotometrically or by HPLC were obtained with reference to calibration curves constructed from identically prepared standards. Dye clearance values were calculated using the total area under the indocyanine green concentration *versus* time curve.

RESULTS AND DISCUSSION

Chromatography—Figure 1 illustrates typical chromatograms for blank rabbit plasma and indocyanine green plasma standards. The total

[12] P-1215, Novopharm.

[13] Carl Zeiss spectrophotometer PMQ II.

[14] Model 351 infusion pump, Sage Instruments.

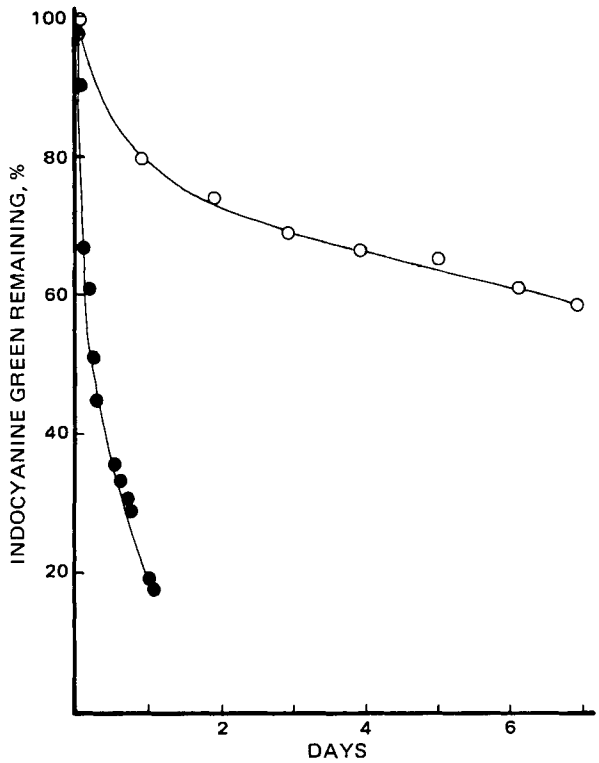

Figure 3—*Degradation profile of aqueous indocyanine green (initial concentration was 20 μg/ml) as examined by HPLC (●) and spectrometric (○) methods.*

retention time was ~7 min, and the quantitative limit of detection for the dye in plasma was 0.4 μg/ml.

The composition of the mobile phase was examined with the following results. The acetonitrile concentration is critical for successful resolution of indocyanine green and diazepam and for optimization of peak shape (Fig. 2). The use of methanol in the ternary solvent system sharpens and reduces the retention time of the internal standard peak without appreciably affecting indocyanine green retention. With respect to the buffer, the salt strength is more important than pH for successful retention of indocyanine green.

The quantitation of indocyanine green in samples varies with the plasma volume (Table I), even when the ratio of precipitant to plasma is constant (1.6:1 v/v). This result may be accounted for by an interfering component in blank rabbit plasma, which exhibits the same retention time as diazepam. Although the diazepam equivalent of this peak only approaches 5% with 0.25-ml samples, the interference is more pronounced with larger sample volumes when the amount of diazepam added remains constant. Therefore, samples with dye concentrations greater than the upper limit of the standard curve (80 μg/ml) were diluted to varying degrees to allow analysis of a constant volume (0.25 ml).

Calibration curves were linear over the concentration range used. The intra-assay coefficient of variation ranged from 2% for 40-μg/ml samples to 8% for 0.4-μg/ml samples (n = 6). The interassay variability was 5–11% for all standards except the 0.4-μg/ml standard, which was 20% (n = 8). The slope variance of standard curves was calculated as 10% (n = 10).

Spectrophotometric Analysis—The lower limit of quantitative detection of indocyanine green in plasma was 2 μg/ml when using 0.25 ml of plasma and the described dilution. The interassay coefficient of variation for all standards was 11–14% (n = 3).

It was reported (18) that different plasma samples yield calibration curves that vary widely, but no explanation for this result has been found. A similar observation was made in this investigation when blank plasma from different rabbits was used. The slopes of calibration curves varied by 32%. This variability is independent of any differences in the inherent absorbance of blank samples at 800 nm. The problem was also not alleviated by protein precipitation using acetonitrile prior to spectrophotometric measurement. Thus, although the source of the variability has not been resolved, it demonstrates a disturbing characteristic of the spectrophotometric method.

Stability Studies of Aqueous Solutions—Once an accurate, reproducible HPLC method for indocyanine green was developed, preliminary studies were conducted to examine the stability of the dye in aqueous solution. Figure 3 illustrates the inadequacy of the spectrophotometric method in the detection of dye degradation in aqueous media. The discrepancy between the two methods results from degradation products of indocyanine green, which absorb a significant amount of light at 770 nm.

Table I—Quantitation Efficiency (Mean ± *SD*) Observed for Indocyanine Green (2 μg) when Added to Plasma Containing 0.735 μg of Diazepam (n = 6)

Plasma Volume, ml	Quantitation Efficiency, %
0.125	91.8 (1.4)
0.25	86.9 (1.8)
0.5	78.5 (2.0)
1.0	72.1 (4.6)

By modifying the mobile phase flow rate, these degradation products can be resolved (Fig. 4). Spectral analysis of the eluate fractions corresponding to these peaks showed that peaks a and b exhibited absorbance at 770 nm. It is unlikely that these peaks represent dye aggregates as described earlier (12, 19), since eluates were below the aggregate-forming concentration (<5 μg/ml) and the ionic strength of the solvent (mobile phase) was constant in all cases. Thus, it was felt that these peaks (Fig. 4) represent products of an irreversible degradation of parent dye. Barbier and DeWeerdt (16) showed two chromatographically distinct species that appear on storage of aqueous dye solution. The "fast" and "slow" fractions exhibited spectral peaks in the visible region at 780 and 783 nm, respectively.

The evidence from these experiments provides another reason why reports using the spectrophotometric method should be questioned.

Dye Stability in Plasma—Standard plasma samples analyzed by the HPLC method exhibited no detectable degradation after 10 weeks of storage at 6°, a finding in agreement with results obtained spectrophotometrically (10, 12).

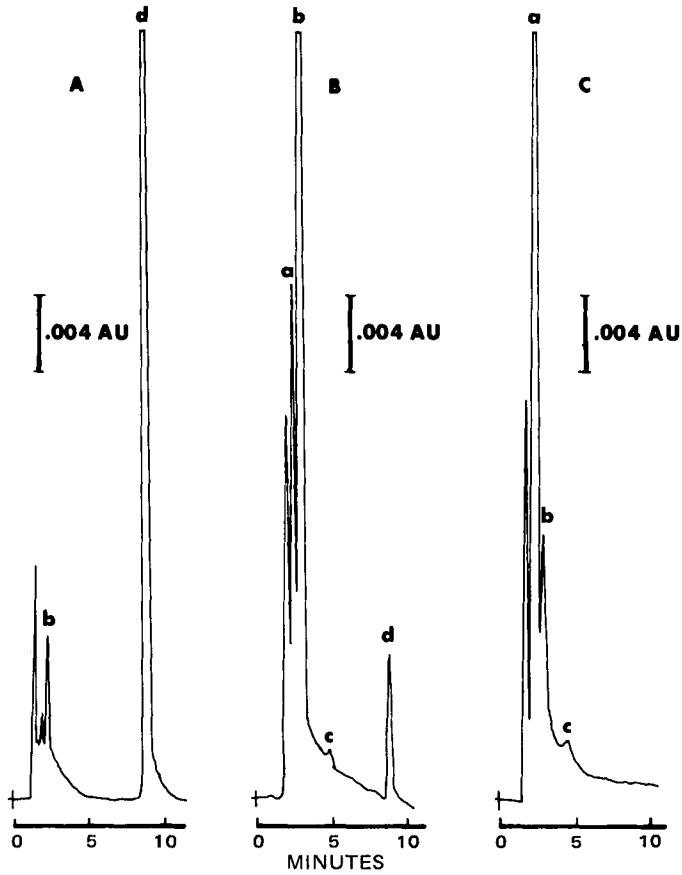

Figure 4—*HPLC chromatograms obtained from aqueous indocyanine green solutions that were freshly prepared (A), 1 day old (B), and 7 days old (C). An RP-18 column (250 × 4.6-mm i.d.) and a mobile phase of 47% acetonitrile–3% methanol–0.05 M phosphate buffer (pH 6) at 1.0 ml/min were employed. Peaks a, b, and c are degradation products of indocyanine green; and peak d is indocyanine green.*

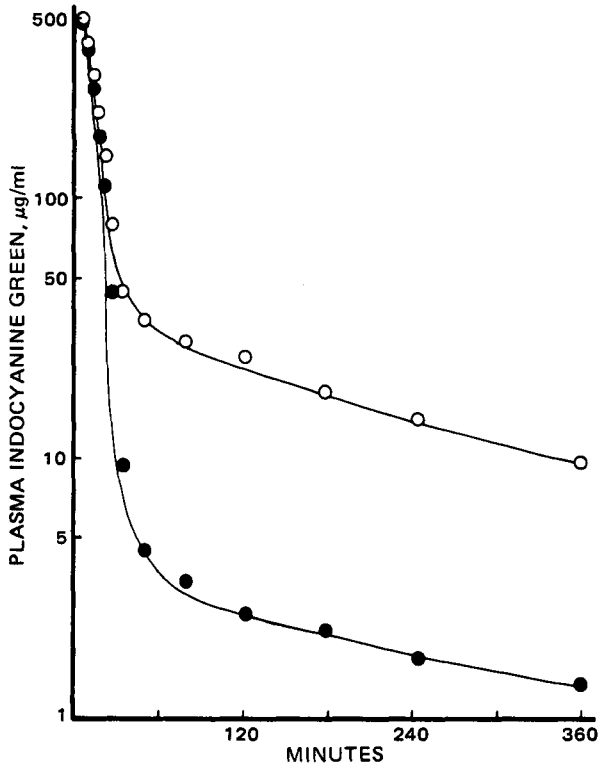

Figure 5—*Plasma indocyanine green observed in a rabbit following a 25.6-mg/kg dose as examined with HPLC (●) and spectrophotometric (○) methods.*

Indocyanine Green *In Vivo* Study—Having established that the HPLC method was more specific for the parent dye in examining aqueous stability, a preliminary study of *in vivo* pharmacokinetics was conducted in a rabbit. Figure 5 illustrates the plasma concentration *versus* time profiles obtained when the same samples were analyzed by the two methods. Initially, indocyanine green concentrations were similar by both procedures. However, after the steep declining phase, their difference became more pronounced. The use of the spectrophotometric method resulted in an erroneously low dye clearance (spectrophotometric method, 1.58 ml/min/kg; HPLC, 2.65 ml/min/kg).

It was questioned whether the observed difference in plasma indocyanine green (Fig. 5) was an artifact or a consequence of the nonspecificity of the spectrophotometric assay. One step in resolving this question was to collect plasma from a rabbit given normal saline rather than indocyanine green. Relative to water, samples collected over 5 hr exhibited an absorbance of 0.0058–0.0067 (790 nm), demonstrating that no time-related changes were found in the inherent absorbance of indocyanine green-free plasma. In view of the plasma stability studies, dye degradation after sample collection was also not responsible for the difference since both analyses were performed on the day of the *in vivo* experiment. It was thus concluded that an *in vivo* degradation product or metabolite of the dye was potentially responsible for the discrepancy seen in Fig. 5.

To evaluate the "metabolite" hypothesis further, the HPLC solvent front eluate was collected for *in vivo* samples, plasma standards, and a plasma blank. It was suspected that the discrepancy noted in Fig. 5 was due to an indocyanine green product eluting in the solvent front (similar to Fig. 4). Spectrophotometric scans of the solvent front eluates of plasma blank and dye standards showed no detectable absorbance in the 600–800-nm region (Fig. 6). However, all solvent front eluates from *in vivo* samples showed some absorbance in this region (Fig. 6). The absorbance pattern was similar to that of parent indocyanine green. However, the ratio of the absorbance at 770 nm to that at 700 nm was 2.4 for the indocyanine green peak eluates and 1.9 for solvent front eluates. For reasons discussed earlier with respect to dye degradation, it is unlikely that the component found in the solvent front eluate represents a dye aggregate. Therefore, it is postulated that indocyanine green undergoes some degradation or metabolism in the body prior to its excretion. The fact that the unknown substance elutes from the HPLC column prior to the parent dye is suggestive of a compound that is more polar than indocyanine green.

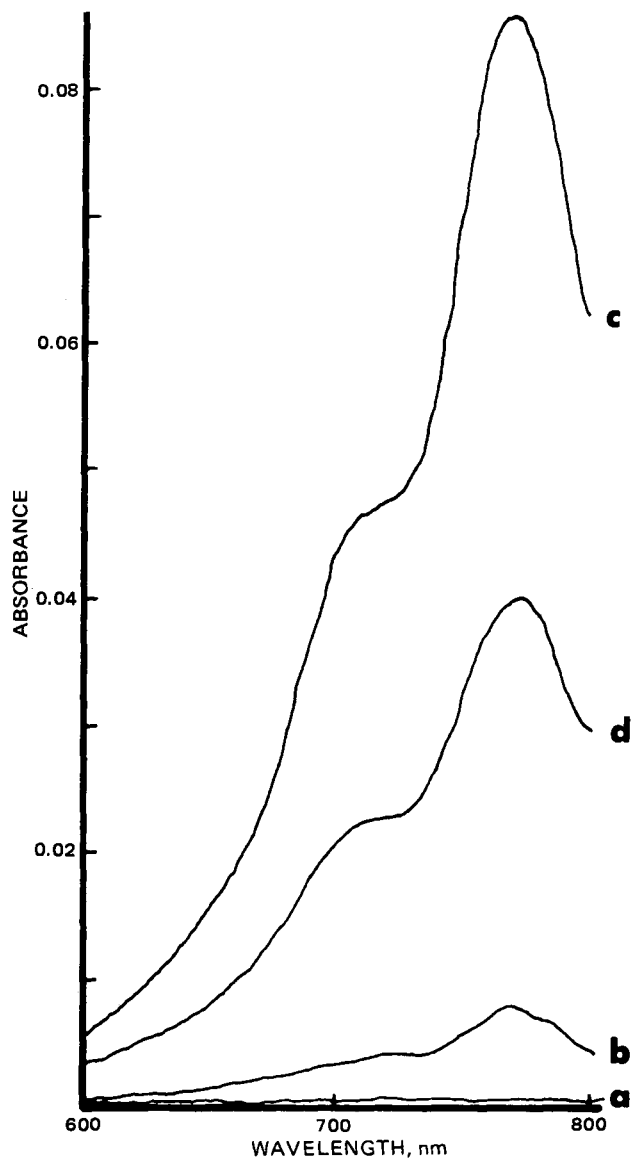

Figure 6—*Spectrophotometric scans of collected HPLC solvent front eluates from processed plasma samples harvested from a rabbit receiving 25.4 mg of indocyanine green/kg. Key: a, instrument baseline (blank solvent in both cells); b, plasma sample 26-min postdose (40 μl of supernate injected); c, plasma sample 120-min postdose (250 μl of supernate injected); and d, plasma sample 360-min postdose (250 μl of supernate injected).*

Only one literature report was found suggesting indocyanine green metabolism. Nambu (20), using ascending chromatography, found three bands in bile samples collected from a dog administered indocyanine green. The three bands exhibited absorbance at 770 nm in a pattern similar to that of the present studies.

The present study demonstrated that the described HPLC method is more specific for indocyanine green then the traditional spectrophotometric methods. This observation demands a reexamination of reports on indocyanine green's stability and *in vivo* pharmacokinetics. Furthermore, the nature of the degradation products/metabolites needs to be elucidated. These aspects are under investigation.

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Analysis of Oral Suspensions Containing Sulfonamides in Combination with Erythromycin Ethylsuccinate

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Abstract □ The sulfonamides and erythromycin ethylsuccinate in combination oral suspensions were determined by high-performance liquid chromatography and automated turbidimetry, respectively. The chromatographic procedure was rapid, specific, and stability-indicating for sulfisoxazole acetyl and the trisulfapyrimidines using a reversed-phase system with UV detection at 254 nm. Erythromycin ethylsuccinate did not interfere with the sulfonamide analysis and these compounds were assayed with relative standard deviations (*RSD*) ranging from ±2.1 to ±3.1%. Erythromycin ethylsuccinate was determined as erythromycin with *RSD* values of ±1.3 or ±3.5% without interference by the sulfonamides present.

Keyphrases □ Sulfonamides—analysis of oral suspensions in combination with erythromycin ethylsuccinate, high-performance liquid chromatography □ Erythromycin ethylsuccinate—analysis of oral suspensions with sulfonamides, high-performance liquid chromatography □ High-performance liquid chromatography—analysis of oral suspensions containing sulfonamides in combination with erythromycin ethylsuccinate

Oral suspensions containing sulfonamides in combination with erythromycin ethylsuccinate have recently been developed for the treatment of acute otitis media. This paper presents the analysis of the sulfonamides and erythromycin ethylsuccinate present in two oral suspensions: erythromycin as erythromycin ethylsuccinate at 200 mg/5 ml and sulfisoxazole as sulfisoxazole acetyl at 600 mg/5 ml (I) and erythromycin as erythromycin ethylsuccinate at 200 mg/5 ml and trisulfapyrimidines: sulfadiazine, sulfamerazine, and sulfamethazine, each at 200 mg/5 ml (II).

Sulfonamide dosage forms are commonly assayed by nitrite titrations or colorimetric methods based on a previous (1) procedure. In mixtures containing more than one sulfonamide or in complex biological matrixes, paper and thin layer chromatography (TLC) have been used to quantitate the individual drugs (2–5). Often these chromatographic separations are followed by the Bratton and Marshall procedure (6–9). The current USP assay (10) of trisulfapyrimidine oral suspensions uses such a procedure and requires several hours to complete.

Use of gas-liquid chromatography (GLC) in the analysis of sulfonamides has been reported (11–14), but derivatization is generally required. High-performance liquid chromatography (HPLC) was used in this study to quantitate the individual sulfonamides. Separations of sulfonamides using ion exchange (15–17), reverse phase (18–24), normal phase (25–28), and ion pairing (29–30) are reported in the recent literature.

Numerous analytical techniques have been reported for the analysis of erythromycin and its various esters. Included are chemical methods based on ultraviolet–visible spectrophotometry (31–34) or fluorometry (35) in addition to GLC (36, 37), TLC (38–41), HPLC (42–45), and microbiological techniques (46–48). Since the microbiological assay is the official methodology required for the determination of erythromycin potency (49), an automated turbidimetric method was employed in this work.

EXPERIMENTAL

Reagents—Acetanilide[1], benzanilide[1], and potassium phosphate[2] (monobasic and dibasic) were obtained commercially and used without further purification. Sulfisoxazole acetyl[3,4], erythromycin base[3], erythromycin ethylsuccinate[5], sulfadiazine[3], sulfamerazine[3], and sulfamethazine[3] were of pharmaceutical quality and were used as received. Acetonitrile[6], chloroform[6], and methanol[6] were HPLC grade. Oral suspensions I[5] and II[5] were prepared from granules prepared in house.

[1] Eastman Kodak Co., Rochester, N.Y.
[2] AR grade, Mallinckrodt, Inc., St. Louis, Mo.
[3] USP Reference Standards, U.S. Pharmacopeia, Rockville, Md.
[4] Hoffmann–LaRoche, Inc., Nutley, N.J.
[5] Abbott Laboratories, North Chicago, Ill.
[6] Burdick & Jackson Laboratories, Muskegon, Mich.

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