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RESEARCH ARTICLES

Quantitation of Lipophilic Chloroethylnitrosourea Cancer Chemotherapeutic Agents

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Received February 27, 1981, from the **Brain Tumor Research Center, Department of Neurological Surgery, School of Medicine, University of California, San Francisco, CA 94143* and the ‡*Department of Medicinal Chemistry, School of Pharmacy, Purdue University, West Lafayette, IN 47907.* Accepted for publication June 2, 1981.

Abstract □ A simple and rapid quantitative method for the derivatization and determination of lipophilic chloroethylnitrosoureas is described. This procedure involves the ether extraction of the chloroethylnitrosourea from plasma and conversion of the parent drug to an *O*-methylcarbamate by reaction in anhydrous methanol. The product *O*-methylcarbamate may be separated with gas chromatography (GC) and detected with nitrogen-specific GC detectors or with mass spectrometry using multiple-ion detection. The lower limit of detection for each method was ~100 ng/ml plasma.

Keyphrases □ Cancer chemotherapeutic agents—lipophilic chloroethylnitrosoureas, quantitation □ Chloroethylnitrosoureas—quantitation by gas chromatography □ Gas chromatography—quantitation of lipophilic chloroethylnitrosourea cancer chemotherapeutic agents

The described assay was applied to 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine, I), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine, II), 1-(2-chloroethyl)-3-(4-*trans*-methylcyclohexyl)-1-nitrosourea (semustine, III), 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitrosourea (IV), and 1-(2-chloroethyl)-3-(4-amino-2-methyl-5-pyrimidinyl)methyl-1-nitrosourea (V)[1]. The clearance curve for IV from rat plasma is presented as an example of the application of this method to the determination of biodistribution parameters.

BACKGROUND

The chloroethylnitrosoureas are a class of highly effective and widely used cancer chemotherapeutic agents (1–3). Analysis of these drugs in plasma at therapeutically significant concentrations has proven to be difficult. Although several methods of analysis have been reported, none are generally applicable to patient pharmacokinetic analyses. As a consequence, only carmustine pharmacokinetics has received detailed study (4).

Chloroethylnitrosoureas are thermally labile and decompose at or below 100° so that the parent compounds cannot be analyzed with gas chromatography (GC) (5). Compounds of this class are readily separated with high-performance liquid chromatography (HPLC) (6); however, the low wavelength and molar absorptivity of the nitrosourea chromophore

[1] These compounds are commonly referred to by their respective code designations BCNU, CCNU, Methyl-CCNU, PCNU, and ACNU.

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(λ_{max} 232 nm, ϵ 6000) causes this approach to lack sensitivity even when variable-wavelength UV detectors are used. An assay limit of 2–5 μg/ml of plasma was found with II (7, 8). Compound V is an exception to this generalization, as the pyrimidinyl substituent is a superior chromophore (9). Both HPLC and GC analyses are hindered by the fact that the chloroethylnitrosourea group is difficult to derivatize.

Radiolabeled chloroethylnitrosoureas, especially I and II, have been used for the measurement of tissue concentrations of parent drug either without separation (10, 11) or after isolation of unchanged drug with TLC (12) or HPLC[2]. This approach is sensitive but the use of radioactive tracers in patients is limited. The colorimetric assay developed by Loo and Dion (13) has a lower detection limit than HPLC but does not possess sufficient sensitivity to assay patient plasma samples. This assay involves the acid-catalyzed decomposition of nitrosourea to nitrous acid which is subsequently trapped and analyzed using the Bratton–Marshall method (14). This approach lacks the specificity necessary to distinguish between parent drug and biologically active metabolites that retain the nitrosourea moiety.

Recently reported polarographic methods have the requisite sensitivity but are susceptible to interference from plasma components and also lack specificity (15). Patient pharmacokinetic studies of carmustine biodistribution have been performed using direct sample insertion chemical-ionization mass spectrometry (5). The method was successful because of the relatively high lipophilicity and volatility of I and is not applicable to less volatile analogs such as II and IV.

The described analytical procedure may be applied to different chloroethylnitrosourea analogs. The parent nonpolar chloroethylnitrosoureas may be extracted from plasma and converted to a stable *O*-methylcarbamate by reaction in anhydrous basic methanol. The *O*-methylcarbamate products may be separated with GC and detected at high sensitivity with multiple-ion detection mass spectrometry or with nitrogen-specific GC detectors.

EXPERIMENTAL

Materials—Reagents used to convert chloroethylnitrosoureas[3] to *O*-methylcarbamates were 0.2 *M* trimethylanilinium hydroxide in anhydrous methanol[4], 10% freshly distilled triethylamine[5] in anhydrous methanol[5], 10% reagent grade pyridine[5] in anhydrous methanol[5], and 2% sodium methoxide in anhydrous methanol prepared by reacting sodium metal with anhydrous methanol.

Instrument Conditions—GC separations of *O*-methylcarbamate derivatives of chloroethylnitrosoureas were performed on instruments equipped with 1.5 m long, 2-mm i.d. glass columns packed with 3% OV-1 on 100–120 mesh Supelcoport[6]. An instrument equipped with a flame-ionization detector[7] and an instrument equipped with a nitrogen-specific detector[8] used helium carrier gas at a flow rate of 40 ml/min. An instrument interfaced to a mass spectrometer[9] operating in the multiple-ion detection mode and equipped with a chemical-ionization source used methane[10] as a combined carrier and reagent gas. In each case, an injection port temperature of 210° was used. Column oven temperatures were varied according to the nitrosourea derivative being analyzed.

Derivatives of I and II were eluted using linear temperature programming with an initial temperature of 70° increasing 4°/min starting at the time of injection. The derivative of I eluted in ~2 min at 80° and II eluted in ~6.5 min at 95°. Isothermal column oven conditions may be used for other derivatives and derivatives of II and III may be eluted at 110° with retention times of ~2.0 and 3.0 min, respectively. Compounds IV and V may be eluted at 160° as *N*-methyl-*O*-methylcarbamates with retention times of ~2.0 and 3.0 min, respectively. GC–mass spectrometric operations were performed at an ion-source pressure of 1.0 mm methane and temperature of 200°.

Extraction and Derivative Formation—Chloroethylnitrosoureas, 0.05–1.0 μg, were added to 1.0 ml of plasma or to 1.0 ml of 0.05 *M* phosphate buffer (pH 7.4) and extracted with 3.0 ml of ether. The 2.0-ml ether layer was removed and placed into a 3.0-ml cone-shaped interior vial[11] containing 1.0 μg of a reference chloroethylnitrosourea. The ether was evaporated to dryness under a nitrogen stream at ambient temperature. Approximately 50 μl of derivatizing reagent was added. Compounds IV and V were derivatized with 0.2 *M* trimethylanilinium hydroxide in anhydrous methanol[4]. Compounds I–III were derivatized with 2% sodium methoxide in anhydrous methanol. The reagent–chloroethylnitrosourea mixture was heated at 50° for 20 min in the capped cone-shaped vial[11]. Approximately 1 μl of this mixture was injected onto the chromatograph column and the peak height ratios were determined. These peak height ratios were compared with those obtained from the analysis of the same amounts of chloroethylnitrosoureas added directly to ether and avoiding the extraction step to determine extraction efficiency.

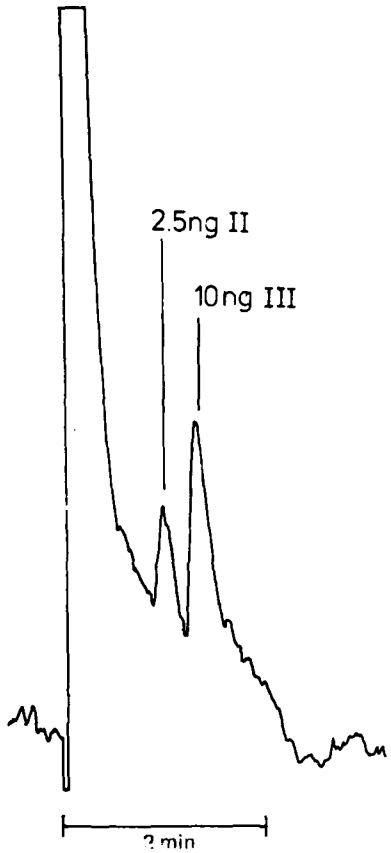


Figure 1—*A chromatogram of O-methylcarbamates derived from II and III. Separations were performed using a 3% OV-1 column at 110° and detected using a nitrogen-specific detector. The amounts injected on-column were calculated from the amounts of II (50 ng) and III (250 ng) extracted from 1 ml of water, derivatized with 20 μl of 2% sodium methoxide in anhydrous methanol and injected assuming 100% conversion at each step.*

Derivatization conditions were optimized by comparison of absolute peak heights obtained from heating derivatizing reagents with chloroethylnitrosoureas at different temperatures and for various times. Optimal peak heights were obtained on heating the reagent mixtures at 50° for 20 min. No decrease in peak heights was observed on heating for longer periods.

Analysis of IV with V as Standard—A 0.1–0.5-ml plasma sample containing IV was mixed[12] with 3.0 ml of ether. The ether (2.0 ml) was removed and placed in a 3.0-ml cone-shaped vial[11] containing 10 μl of a 100 μg/ml stock solution of V in ether (1.0 μg, 3.5 nmoles). The ether was evaporated to dryness under a nitrogen stream at ambient temperature. Approximately 20 μl of 0.2 *M* trimethylanilinium hydroxide in anhydrous methanol[4] was added, mixed, and heated at 50° for 20 min in a capped cone-shaped vial. Approximately 1.0 μl of this mixture was injected onto the column. The eluted peaks were detected with mass spectrometry using multiple-ion detection of ions *m/z* 183.1 for IV and *m/z* 191.1 for V-derived products. The peak height ratio of 183.1/193.1 was determined, and the plasma IV concentration was calculated by reference to a standard curve obtained using the described procedure with known concentrations of IV in plasma. Alternatively, the eluted GC peaks

[2] V. A. Levin and P. Kabra, unpublished results.

[3] Compounds I–IV were obtained from Dr. Robert Engle of the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute and V was obtained from Sayko Co. Ltd., Japan.

[4] Methelute, Pierce Chemical Co.

[5] J. T. Baker Chemical Co.

[6] Supelco, Inc.

[7] Varian Associates model 2100.

[8] Perkin-Elmer model L-14.

[9] Finnigan Corp. model 3200.

[10] Ultra-high purity, Matheson.

[11] American Scientific Products.

[12] Vortex mixer, VWR Scientific Inc.

may be detected with a nitrogen-specific detector and the peak height ratios determined. The plasma concentration was calculated by reference to a standard curve obtained using this detection method.

Analysis of Other Chloroethylnitrosoureas—The described procedure for the analysis of IV may be followed for the analysis of plasma containing V by using IV as a standard.

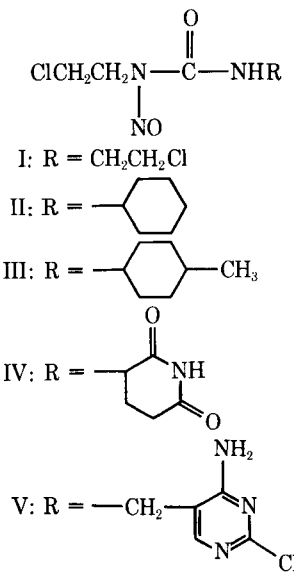
Compounds I–III may be quantified by mixing 0.1–0.5 ml of plasma containing a chloroethylnitrosourea with 3.0 ml of ether. The ether (2.0 ml) was removed and placed in a 3.0-ml cone-shaped vial containing 1.0 μg of a standard chloroethylnitrosourea. Recommended standards are II for quantification of I or III, and III for quantification of II. Approximately 20 μg of 2% sodium methoxide in anhydrous methanol was added, mixed, and heated at 50° for 20 min in a capped cone-shaped vial. Approximately 1.0 μl of this mixture was injected onto the column.

The eluted peaks were detected with mass spectrometry using multiple-ion detection of ions *m/z* 138.1 or 102.1 for I, *m/z* 158.1 for II, and *m/z* 172.1 for III-derived products. The respective peak height ratios may be determined and used to calculate plasma concentration by reference to a standard curve obtained using the described procedure for analysis of known amounts of sample and reference chloroethylnitrosourea. Eluted GC peaks may be detected using a nitrogen-specific GC detector to obtain the corresponding peak height ratios which may also be used to calculate plasma concentrations.

Clearance from Rat Plasma—Male Fisher-344 rats, 230–290 g, were anesthetized by an intraperitoneal injection of pentobarbital and catheters were inserted into the saphenous artery and vein. After intravenous administration of 100 USP units of heparin sodium, rats were administered 22.2–22.9 mg of IV/kg iv for 20 sec. Samples of arterial blood were removed through the arterial catheter at 1, 2, 4, 6, 10, 15, 30, 60, 90, 120, and 180 min following intravenous administration. Individual 0.2–0.5-ml samples were placed in 1.5-ml capped micro test tubes and centrifuged[13] immediately at 8000×*g* for 30 sec. A known volume (0.1–0.3 ml) of plasma was removed and analyzed as described.

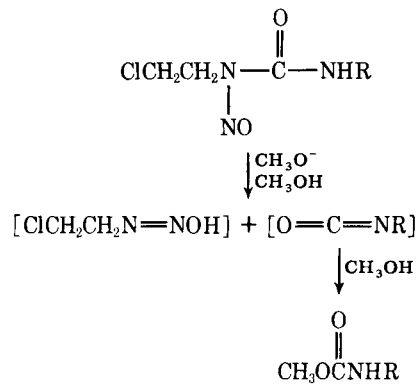
RESULTS AND DISCUSSION

Clinically studied chloroethylnitrosoureas are a class of drugs that share a common reactive nitrosourea moiety but may contain a variety of *N*-substituents, some of which are shown in Scheme I. The described assay employs the known base-catalyzed decomposition reaction of 3-substituted 1-(2-chloroethyl)-1-nitrosoureas to 2-chloroethylazohydroxide and *N*-substituted isocyanates (16–21). In this assay, the decomposition reaction is carried out in anhydrous methanol where the intermediate isocyanates react to yield *N*-substituted-*O*-methylcarbamates (22) as shown in Scheme II. The carbamate products are formed in >90% yield and retain the characteristic R-substituent of the different chloroethylnitrosoureas and their biologically active metabolites (23, 24). The *O*-methylcarbamate derivatives are readily separated by GC and may be detected using a variety of methods.



Scheme I—The structure of lipophilic chloroethylnitrosourea cancer chemotherapeutic agents.

[13] Eppendorf model 5412 centrifuge.



Scheme II—The reaction of chloroethylnitrosourea in basic anhydrous methanol leads to the formation of 2-chloroethyldiazotate and substituted isocyanate. The isocyanate reacts with solvent methanol to give a stable O-methylcarbamate derivative.

The assay procedure requires that the nitrosourea be extracted from plasma. All of the analogs studied (Scheme I) are lipophilic and are readily extracted from aqueous media by organic solvents. The least lipophilic analog, IV, has an octanol/water partition coefficient of 2.3. The extraction efficiency of this compound from 0.5 ml of plasma by a sixfold volume excess of ether is high. When ratios of known amounts of IV to V are plotted against observed peak height ratios, the same slope is obtained from the analyses of known amounts of both compounds following extraction of both components from plasma, and following extraction of IV from plasma using V as a standard. These results confirm that both compounds may be extracted from 0.5 ml of plasma by 3.0 ml of ether in >90% efficiency.

The decomposition of chloroethylnitrosoureas to alkylisocyanates is catalyzed by organic and inorganic bases. Although the choice of catalyst is not critical, 2% sodium methoxide in anhydrous methanol is a convenient reagent compatible with GC nitrogen-specific detectors and with mass spectrometric detection under chemical-ionization conditions. This reagent may be used for the analysis of IV and V as well as the other chloroethylnitrosoureas listed in Scheme I. Trimethylanilinium hydroxide in anhydrous methanol may be used for the analysis of IV and

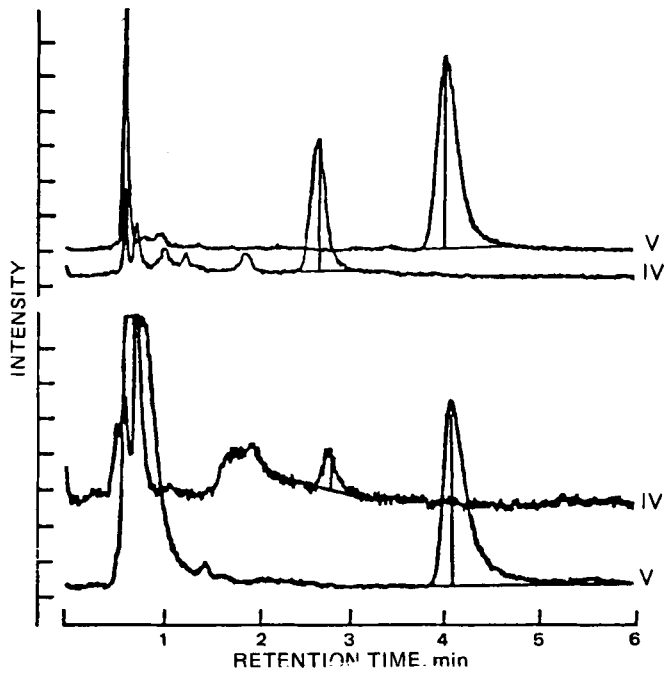


Figure 2—*GC–mass spectrometric selected ion traces for IV and V obtained from rat plasma following the described assay procedure for IV using V as a standard. The upper portion of the figure is the analysis of V in rat plasma following intravenous administration of a 22.5-mg/kg bolus of IV. The lower portion shows the data output operating near the limit of detection in which an estimated 2.0 ng of derivative was injected on-column.*

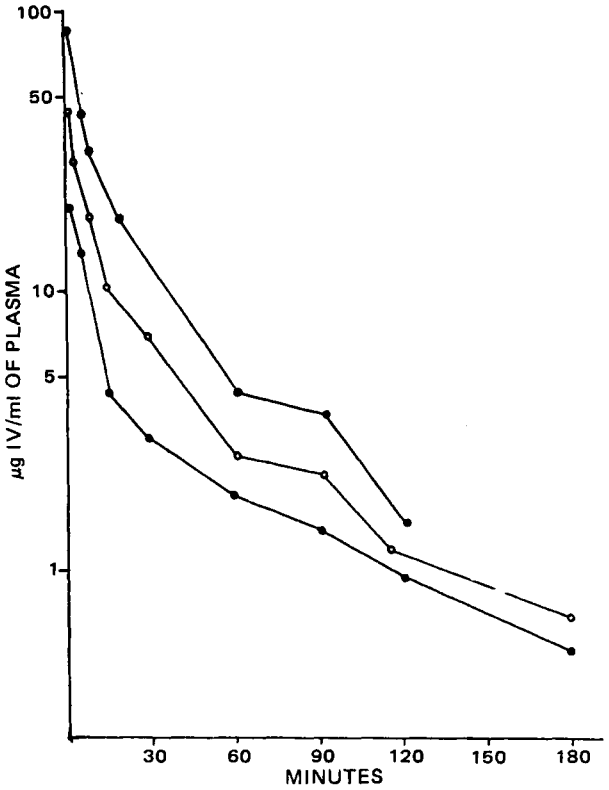


Figure 3—*Plasma clearance curves obtained following the intravenous administration of 22.5-mg/kg bolus of IV to 230–290-g male Fisher-344 rats. Compound IV was analyzed following the procedure using V as a standard. The GC eluate was monitored using mass spectrometry multiple-ion detection.*

V but is not recommended for the analysis of other analogs. This reagent acts as an on-column methylating agent as well as a basic catalyst.

Reaction of a chloroethylnitrosourea at 50° for 20 min in the reagent forms the *O*-methylcarbamate (Scheme II). Injection of the mixture onto the GC column at an injection port temperature of 210° pyrolyzes the trimethylanilinium ion and transfers a methyl group onto the nitrogen of the *O*-methylcarbamate. The resulting *N*,*N*-disubstituted carbamate has good GC characteristics. *N*-Methylation also facilitates chemical-ionization induced fragmentation to the $(MH-HOCH_3)^+$ ion, thereby concentrating the ion current at a single mass and improving detection sensitivity. However, the on-column methylation of I–III and derived *O*-methylcarbamates is not complete under these conditions, so that the use of this reagent leads to partial methylation and generation of two GC peaks for each compound.

GC peak detection was performed using flame-ionization detectors, nitrogen-specific detectors, and multiple-ion detection mass spectrometry. Flame-ionization detection of plasma-extracted I has a detection limit of 100 ng injected on-column. This rather high limit is due, in part, to the poor separation between the volatile I derivative and plasma contaminants. Although the flame-ionization detection limit may be lower for other chloroethylnitrosourea analogs and GC column packings, this method was not pursued because the nitrogen-specific GC detector proved to be a much more sensitive and selective device with approximately the same cost and complexity as the flame-ionization detector.

Figure 1 shows a chromatogram obtained from the rapid elution of 2.5 ng of II- and 10.0 ng of III-derived products injected on-column. This detection limit is well within that needed to perform pharmacokinetic studies and is similar to that observed using mass spectrometric detection. The values for on-column injection were calculated from the amounts of II and III extraction from water, derivatized, and injected, assuming 100% efficiency at each step. The selectivity of the nitrogen-specific detector for compounds containing the C–N bond provides chromatograms of neutral plasma ether extracts that are relatively free from interfering peaks.

Multiple-ion mass spectrometric monitoring of GC peaks is a sensitive and selective detection method. A GC-selected ion trace of IV and V derivatives where IV was extracted from plasma using V as a standard is shown in Fig. 2. The monitored ions are the *N*-methyl-*O*-methylcarbamate derivative $(MH-HOCH_3)^+$ fragment ions *m/z* 183.1 for IV and 193.1 for V. The upper portion of Fig. 2 shows the analysis of a rat plasma sample following administration of IV. The lower portion shows the detection limit of IV at 2 ng injected on-column. This amount was calculated from the amount of IV added to plasma and injected on-column assuming 100% efficiency of extraction, derivatization, and sample transfer. The actual amount of *O*-methylcarbamate injected may be less.

Table I—Plasma Clearance Pharmacokinetic Parameters Calculated [a] from the Clearance Curves Obtained Following a 22.5-mg/kg iv Bolus Dose of IV to 230–290-g Male Fisher-344 Rats

V_1	0.65 (0.36)	liters/kg
V_2	1.07 (0.70)	liters/kg
V_{dss}	1.72 (0.79)	liters/kg
k_{10}	0.061 (0.015)	min^{-1}
k_{12}	0.080 (0.039)	min^{-1}
k_{21}	0.051 (0.014)	min^{-1}
AUC	508 (111)	μg min/ml
Clearance	0.036 (0.011)	liters/min

[a] The data were fit to a two-compartment open model (25). Values presented are the mean ±*SD* of five separate experiments.

The described detection method was used to measure the clearance of IV from rat plasma following intravenous administration of a 22.5 mg/kg bolus. The detection limit in rat plasma was 100 ng/ml using 300-μl plasma sample volumes. Clearance curves were obtained for five animals. Three of these curves are shown (Fig. 3). The clearance curves were fitted to two exponentials using a computer program for nonlinear iterative curve fitting. The data were further analyzed by a two-compartment model for biodistribution (25). Mean pharmacokinetic parameters calculated from the five clearance curves (Table I) illustrate the suitability of the described analytical method for the determination of pharmacokinetic parameters.

Comparison of the biodistribution parameters of IV with those of the more lipophilic analog I (26) show that the volume of distribution and compartment volumes are comparable. The major difference between the two agents is that the area under the clearance curve for I, *AUC* = 359 ± 68 μg min/ml, following administration of a 26 mg/kg iv bolus to male Fisher-344 rats, is significantly less than that for IV. The importance of a prolonged presence of parent drug in plasma is not clear in the case of the chloroethylnitrosoureas because the parent agent must be chemically converted to reactive intermediates and, therefore, cleared from plasma to be active.

CONCLUSIONS

The described assay leads to the formation of chloroethylnitrosourea derivatives that retain the R group characteristic of the analogs in this series. As a consequence, this method has the potential to be applied to the analysis of biologically active hydroxylated II and III metabolites. For example, hydroxylated II metabolites may be extracted from plasma or incubation medium with ether (27) and analyzed by the described procedures since ring-hydroxylated II analogs can be separated as *O*-methylcarbamates on OV-1 GC columns[14].

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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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