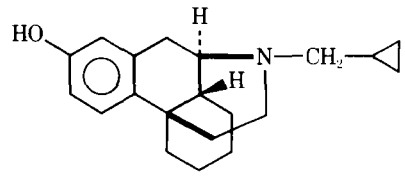
GLC Determination of *l*-2-Hydroxy-*N*-cyclopropylmethylmorphinan in Plasma and Urine

ROBERT E. WEINFELD, ALICE HOLAZO, and STANLEY A. KAPLAN ˣ

Abstract □ A specific method was developed for the determination of *l*-2-hydroxy-*N*-cyclopropylmethylmorphinan in plasma and urine by GLC, using flame-ionization detection. The method involves the extraction of the compound into ether from plasma or urine at pH 7.4, followed by back-extraction into 1 *N* HCl. The acid phase is ether washed and made alkaline, and the compound is reextracted into ether. The ether is evaporated to dryness, the residue is dissolved in methanol, and an aliquot is analyzed by GLC. The same method is applicable to plasma and urine samples following deconjugation of the compound with glucuronidase–sulfatase. The overall recovery is 93.1 ± 9.4% (*SD*) in the concentration range of 0.020–2.0 μg/ml. The method was successfully applied to plasma and urine specimens obtained after administering single 25-mg oral doses to humans.

Keyphrases □ *l*-2-Hydroxy-*N*-cyclopropylmethylmorphinan—GLC analysis, human plasma and urine □ GLC—analysis, *l*-2-hydroxy-*N*-cyclopropylmethylmorphinan in human plasma and urine □ Morphinan, substituted—*l*-2-hydroxy-*N*-cyclopropylmethylmorphinan, GLC analysis in human plasma and urine

Studies on the metabolism of the substituted morphinan, *l*-2-hydroxy-*N*-cyclopropylmethylmorphinan (I), in the dog using ^{14}C-labeled compound indicated that, following 50 mg/kg po, the major urinary metabolite identified was a glucuronide conjugate of I. This compound accounted for approximately 35% of the administered dose[1]. Only about 2% of the dose was recovered as unchanged I. Two other isolated urinary metabolites were identified by GLC–mass spectrometry (1) as the *N*-desalkyl (II) and 7-hydroxy (III) analogs, accounting for 0.5 and 4.3% of the dose, respectively. Most carbon-14 in the dog plasma was present as a conjugate of I, which can be enzymatically released by incubation with glucuronidase.



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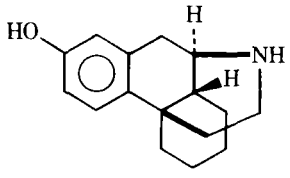
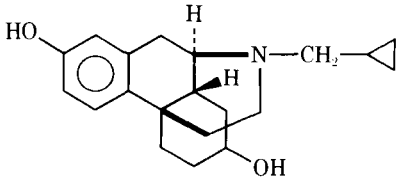


II



III

The use of flame-ionization detection in the GLC analysis of morphines and morphinans in biological fluids is well documented (2, 3). This report describes a sensitive and specific flame-ionization GLC assay procedure for the determination of I and its glucuronide or sulfate conjugate(s) in human plasma and urine.

EXPERIMENTAL

Reagents—All reagents were analytical reagent grade except where noted. All inorganic reagents were prepared using double-distilled water. These reagents included Dulbecco's sodium phosphate-buffered saline[2], pH 7.4, diluted 1:10 for a working solution; 1 *M* acetate buffer, pH 5, prepared by combining 37 parts of 1 *M* sodium acetate and 13 parts of 1 *M* acetic acid; 1 *N* HCl; and 1 *N* NaOH. Anhydrous ether[3], absolute methanol[4], and glucuronidase–sulfatase diagnostic reagent[5] also were used.

Parameters for GLC Analysis—A gas chromatograph[6] equipped with a flame-ionization detector was used. The column was U-shaped, 1.2 m (4 ft) × 4 mm i.d., borosilicate glass, containing a pretested preparation of 3% OV-17 on 60–80-mesh Gas Chrom Q[7]. The temperature settings were: oven, 230°; injection port, 275°; and detector, 300°. The gas flow rates were: carrier (helium)[8], 50 ml/min; hydrogen[8], 100 ml/min; and air, 400 ml/min. The electrometer sensitivity was 1 × 16 (corresponding to 6.4×10^{-11} amp for full-scale deflection on a 1.0-mv recorder[9]). Under these conditions, the retention time, R_t, of I was 7.5–8.0 min, with 400 ng (0.4 μg) injected giving nearly full-scale response.

Preparation of Standard Solutions of I—Weigh 5.60 mg of the hydrochloride salt of I, equivalent to 5.00 mg of the free base, into a 5-ml volumetric flask and dissolve it in absolute methanol to yield a stock solution equivalent to 1 mg of I/ml. Prepare working standard solutions to contain 0.05, 0.1, 0.2, 0.3, and 0.4 μg/10 μl in methanol.

Aliquots of these working standard solutions are used to establish a GLC calibration curve and for addition to plasma and urine as internal standards for the determination of percent recovery.

Experimental Procedure for "Free" I in Plasma and Urine—*Plasma*—Pipet 2.0 ml of plasma and 2.0 ml of phosphate-buffered saline working solution into a 15-ml centrifuge tube. Mix well and extract twice with 5-ml portions of ether, seal the tube with a tetrafluoroethylene[10] stopper, and shake for 10 min on a reciprocating shaker[11]. Centrifuge for 10 min at 2000 rpm in a refrigerated centrifuge[12] at 10°. Transfer the ether layer after each centrifuge step and

[1] J. J. Kamm, A. Szuna, J. Carbone, J. Cheripko, and C. Coutinho, unpublished data.

[2] Catalog No. 0848, Grand Island Biological Co., Grand Island, N.Y.
[3] Catalog No. A-412, Mallinckrodt Chemical Works, St. Louis, Mo.
[4] Fisher Scientific Co., Fairlawn, N.J.
[5] Glusulase (197,173 units of glucuronidase/ml plus 102,425 units of sulfatase/ml), Endo Laboratories, Garden City, N.Y.
[6] Model 402, Hewlett-Packard Co., Avondale, Pa.
[7] Applied Science Laboratories, State College, Pa.
[8] Matheson.
[9] Model 7127A, Hewlett-Packard, Avondale, Pa.
[10] Chemware, Chemplast, Inc., Wayne, N.J.
[11] Model 6000, Eberbach Corp., Ann Arbor, Mich.
[12] Model PR-J, IEC, Needham Heights, Mass.

Table I—Plasma Levels of Free and Total I following 25-mg Single Oral Dose in Human (Subject 1)

Specimen Time, hr	Micrograms per Milliliter of Plasma		
	Total I[a]	Free I	Conjugated I (by Difference)
0	—	—	—
0.5	0.146	0.028	0.118
1	0.219	0.048	0.171
2	0.183	0.061	0.122
4	0.177	0.068	0.109
6	0.091	0.047	0.044
8	0.061	0.023	0.038
12	n.d.[b]	0.023	—
24	n.d.	(0.009)[c]	—

[a] Analyzed by GLC method following glucuronidase–sulfatase treatment. [b] n.d. = not detectable. [c] Estimated value; trace level by GLC.

combine in a 15-ml centrifuge tube. Add 2 ml of 1.0 *N* HCl to the combined ether extracts, shake for 10 min, and centrifuge for 5 min.

Aspirate the ether phase, taking care not to remove any of the acid phase. Wash the acid extract twice with 10 ml of ether by shaking for 10 min and centrifuging for 5 min. Aspirate and discard the ether layer after each washing. Add sufficient 1.0 *N* NaOH to the acid layer to adjust the pH to 8–9 (about 2 ml). Mix well and extract the pH-adjusted solution twice with 5 ml of ether, shaking for 10 min and centrifuging for 5 min. Combine the ether extracts in a clean 15-ml centrifuge tube and evaporate to dryness in a 40° water bath under a gentle stream of nitrogen. Vacuum desiccate the residue for 15 min (over sodium hydroxide pellets) to remove all traces of moisture. Reconstitute the residue in 100 μl of methanol by vigorous mixing on a mechanical mixer[13]. Inject 10 μl into the gas chromatograph.

Along with the unknown specimens, process internal standards of I corresponding to 0.5, 1.0, 2.0, and 4.0 μg. Prepare these standards by evaporating aliquots of the working standard solutions in 15-ml centrifuge tubes under a nitrogen stream in a 40° water bath. Add to each tube 2 ml of control human plasma taken from each subject prior to drug administration.

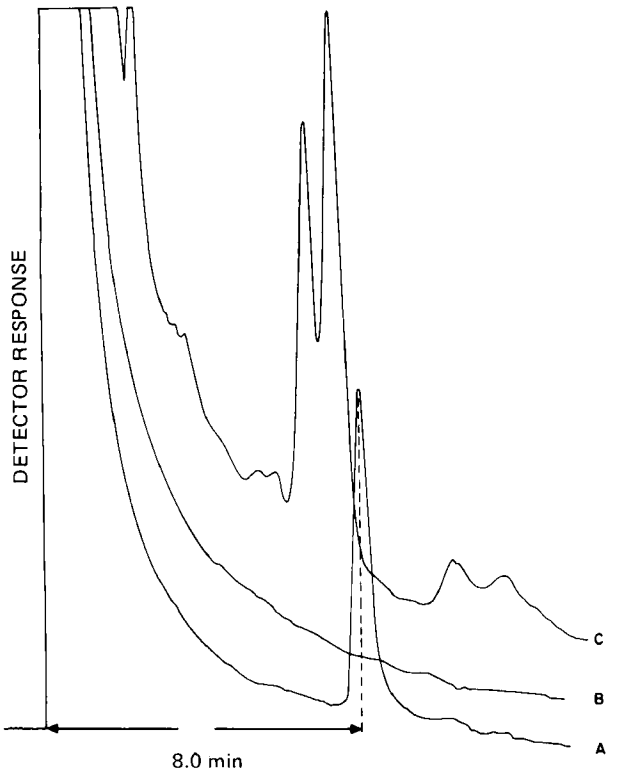


Figure 1—*Chromatograms of: A, authentic standard of I, 200 ng injected; B, control urine following enzymatic hydrolysis and acid cleanup procedure; and C, control urine following enzymatic hydrolysis and no cleanup (10 μl of 100-μl sample injected).*

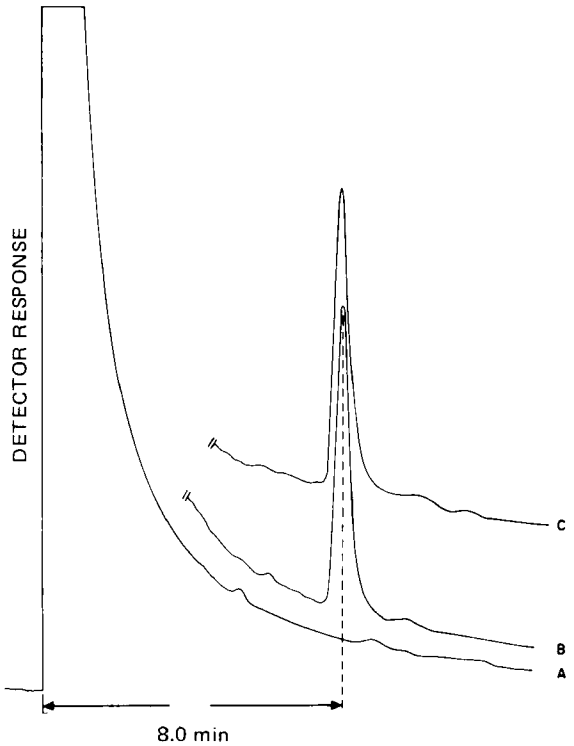


Figure 2—*Chromatograms of: A, control plasma extract; B, internal standard of 2.0 μg of I recovered from control plasma; and C, authentic standard of 200 ng of I injected (10 μl of 100-μl sample injected).*

Urine—Pipet 5 ml of urine into a 50-ml centrifuge tube and adjust to pH 7.4 with 1 *N* NaOH, using a pH meter. Add 5 ml of phosphate-buffered saline working solution, mix well, add 15 ml of ether, and seal the tube with a tetrafluoroethylene stopper. Shake for 10 min and then centrifuge for 5 min at 2000 rpm in a refrigerated centrifuge at 10°. Transfer the ether layer to a fresh 50-ml centrifuge tube and reextract urine with an additional 15 ml of ether. Combine the ether extracts and add 2.0 ml of 1 *N* HCl. Follow the procedure as described for plasma, but wash the acid layer three times with 10 ml of ether. Include a series of internal standards, prepared in the same concentration range as described in the plasma procedure.

Experimental Procedure for "Total" I in Plasma and Urine (I plus Conjugated I)—*Plasma*—Pipet 2.0 ml of plasma into a 25-ml conical centrifuge tube and adjust the pH to 5.0 with 1 *M* acetic acid. Add 0.2 ml of 1 *M* acetate buffer (pH 5), mix, and then add glucuronidase–sulfatase reagent (use 1% of the total aqueous volume after buffer addition). Mix well, stopper loosely with cotton, and incubate overnight (at least 16 hr) at 37° with gentle shaking in a metabolic shaking incubator[14]. Following incubation, adjust the hydrolysate to pH 7.4 with 1 *N* NaOH, add 2 ml of phosphate-buffered saline working solution, mix, and extract twice with 5-ml portions of ether. Then follow the procedure described for I in plasma.

Along with the unknown specimens, process internal standards of I. Prepare these standards by evaporating aliquots of the working standard solutions, corresponding to 0.5, 1.0, 2.0, and 4.0 μg of I, in 25-ml centrifuge tubes under a nitrogen stream in a 40° water bath. Add 2 ml of enzymatically hydrolyzed control (drug-free) plasma to each tube.

Urine—Transfer 1.0 ml of urine into a 25-ml conical centrifuge tube and adjust the pH to 5.0 with 1 *M* acetic acid. Add 0.1 ml of 1 *M* acetate buffer (pH 5), mix, and add glucuronidase–sulfatase reagent (use

[13] Super Mixer, Lab-Line, Melrose Park, Ill.

[14] Precision-Dubnoff, Precision Scientific Co.

Table II—Urinary Concentrations of Free and Total I in Two Subjects following a Single Oral Dose of 25 mg

Subject	Time Period, hr	Percent of Dose, Free	Total I, μg/ml	Total I, mg/hr	Percent of Dose, Total
2	Predrug	—	—	—	—
	0–2		6.5	0.55	4.4
	2–4		25.1	0.29	2.3
	4–6	3.4	23.8	1.01	8.1
	6–8		19.8	0.49	3.9
	8–12		11.0	0.36	5.8
	12–24		1.7	0.18	8.6
	24–48	1.7	3.5	0.11	10.6
(Cumulative)	0–48	5.1	—	—	43.7
3	Predrug	—	—	—	—
	0–2	—	2.1	0.60	4.8
	2–4	—	2.0	0.73	5.8
	4–6	—	9.1	1.04	8.3
	6–8	—	13.3	0.67	5.4
	8–12	—	15.4	0.50	8.0
	12–24	—	3.2	0.24	11.5
	24–48	—	0.9	0.05	4.8
(Cumulative)	0–48	—	—	—	48.6

1% of the total aqueous volume after buffer addition). Mix well, stopper loosely with cotton, and incubate overnight (at least 16 hr) at 37° with gentle shaking in a metabolic shaking incubator. Following incubation, adjust the hydrolysate to pH 7.4 with 1.0 *N* NaOH, add 1 ml of phosphate-buffered saline working solution, mix, and extract twice with 5-ml portions of ether. Then follow the procedure described for free I in urine.

Along with the unknown specimens, process internal standards of I as described in the procedure for total I in plasma; use 1 ml of enzymatically hydrolyzed control urine for each concentration of internal standard used.

Quantitation of I by Flame-Ionization GLC Analysis—The peak height of I in each sample chromatographed is determined by drawing a tangent to the baseline under the peak and measuring the height in centimeters. Peaks that are off scale must be rechromatographed or the electrometer sensitivity must be attenuated to bring the peak on scale for measurement.

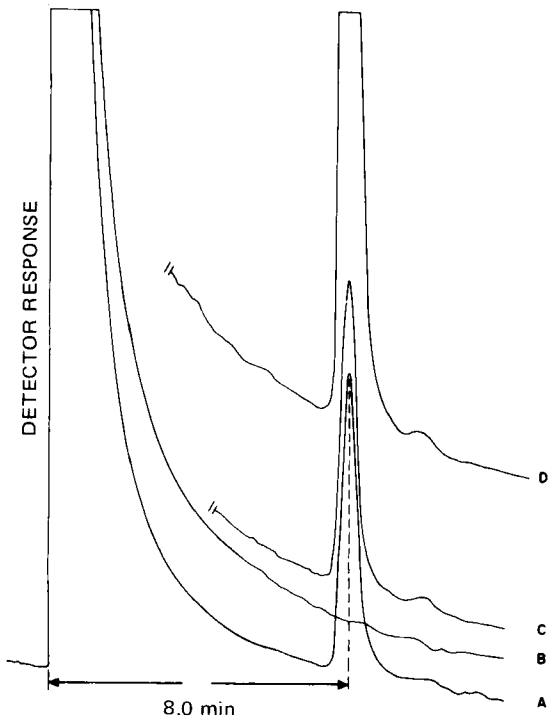


Figure 3—*Chromatograms of: A, authentic standard of 200 ng of I injected; B, control urine extract; C, internal standard of 2.0 μg of I recovered from control urine; and D, patient urine extract following enzymatic deconjugation after 25-mg oral dose of I (10 μl of 100-μl sample injected).*

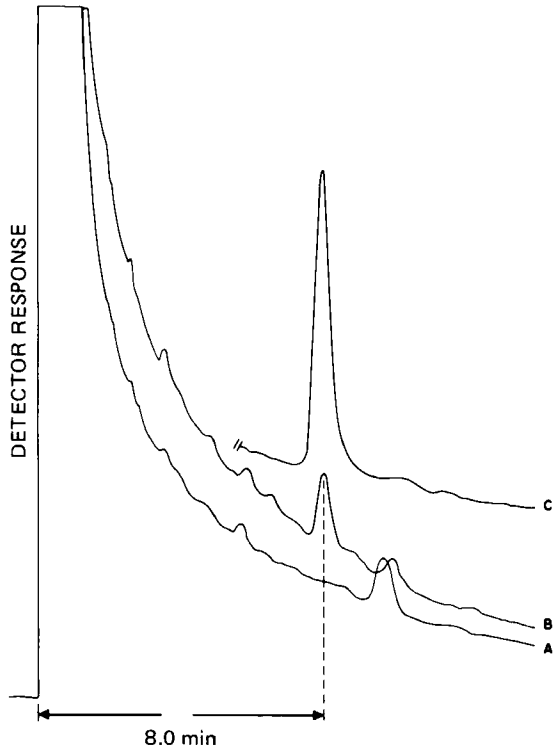


Figure 4—*Chromatograms of: A, control plasma extract injecting 10 of 25 μl; B, patient plasma extract following 25-mg oral dose of I, injecting 10 of 25 μl; and C, authentic standard of 200 ng of I injected.*

A calibration curve of the detector response to I is prepared by plotting peak height *versus* concentration of I injected. The calibration curve of external standards is used to determine the linear response of the detector, the stability of the GLC system, and the percent recovery of added internal standards. The internal standards are used to prepare a calibration curve and for the quantitation of the concentration of I in plasma or urine. Such calibration curves (external and internal) are run with each set of unknowns analyzed. Unknowns are read directly (by interpolation) from the calibration curve to determine the concentration per aliquot injected based on the external standard calibration curve or are read directly from the internal standard calibration curve. The limit of sensitivity is 0.050 μg/ml of biological fluid (using 2 ml for analysis) when injecting 10 μl of the 100 μl of methanol from the reconstituted sample residues for GLC measurement.

If low plasma levels are anticipated, it may be necessary to reconstitute the residue in 25 μl of methanol and inject 10 μl for analysis. The limit of sensitivity is approximately 0.020 μg/ml of biological fluid under these conditions.

The amount of conjugated I per milliliter of plasma or urine is the difference between "total" I and directly extractable I per milliliter measured in each sample. The overall percent recovery of I added to plasma or urine over the range of 0.050–0.400 μg was determined to be 93.1 ± 9.4% (*SD*).

RESULTS AND DISCUSSION

The response of I by flame-ionization detection, using a 1.2-m glass column packed with the phenyl methyl silicone phase (OV-17), was sensitive, reproducible, and linear at levels as low as 20 ng injected. This response to the intact compound was used as the basis for the development of the quantitative determination of I in biological fluids. The extraction of I from plasma and urine at pH 7.4 with ether was quantitative (>93% recovery). Direct GLC analysis could not be performed on the initial ether extract residues without further cleanup, because endogenous impurities extracted from plasma and urine gave interfering peaks.

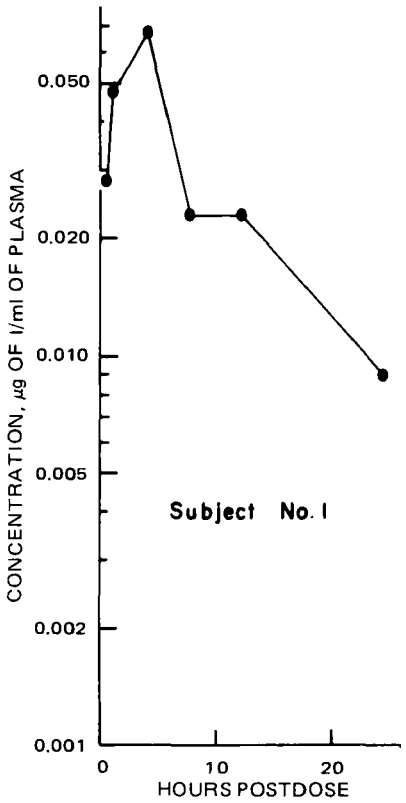


Figure 5—*Plasma levels of free I in a human subject following a single 25-mg oral dose of I, as determined by GLC.*

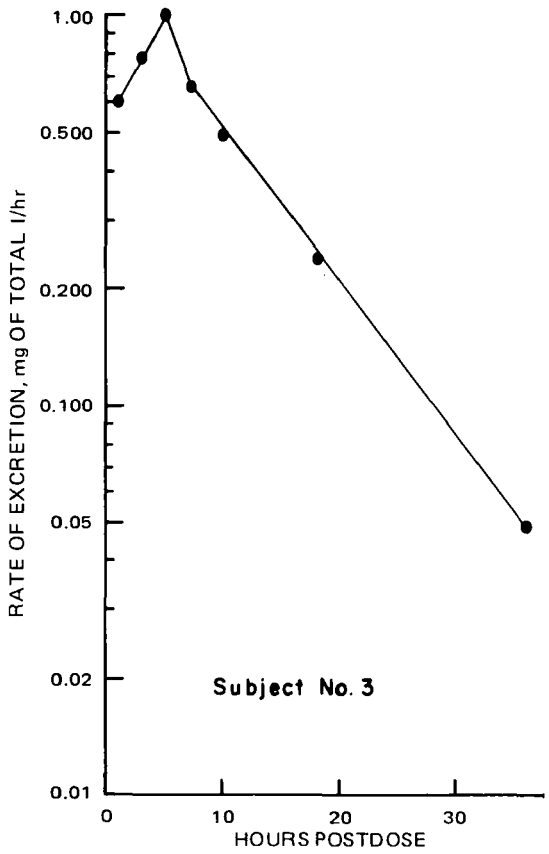


Figure 7—*Urinary excretion rates of total I in a human subject following a single 25-mg oral dose of I.*

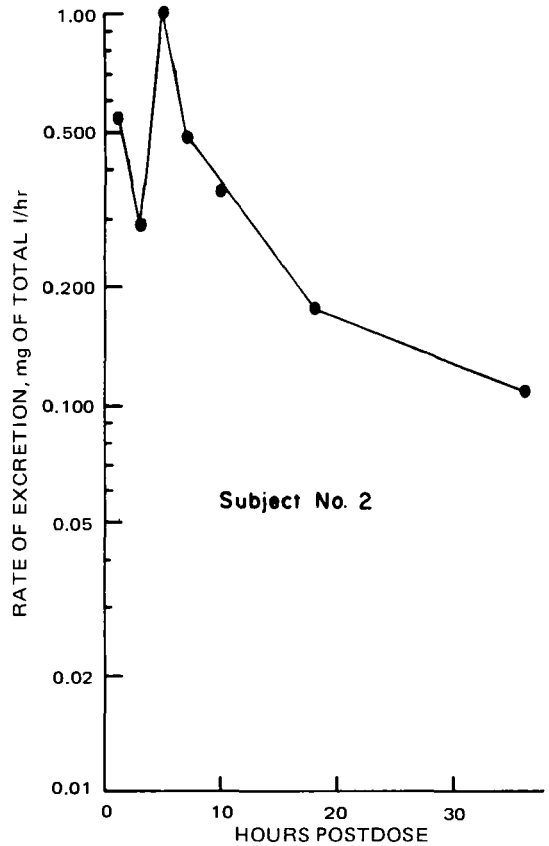


Figure 6—*Urinary excretion rates of total I in a human subject following a single 25-mg oral dose of I.*

This interference was especially evident with urine specimens that were pretreated with glucuronidase–sulfatase, extracted with ether, and analyzed by GLC. The chromatograms showed a major interfering peak eluting near the retention time corresponding to I under the GLC conditions described. These interfering urinary components necessitated additional cleanup of the sample extract, including the acid cleanup step prior to GLC analysis. The resulting chromatograms of control urine (drug free) indicated the absence of the previously interfering peaks (Fig. 1). Plasma samples analyzed by the described method showed no interfering peaks at the retention time, R_t, of I (Fig. 2).

Analysis for total I in urine indicated the excretion of appreciable amounts of I glucuronide or sulfate conjugate(s), determined by the amount of I liberated following glucuronidase–sulfatase incubation, extraction, cleanup, and GLC analysis (Fig. 3).

Adequate sensitivity (0.050 μg of I/ml of plasma) was obtained by injecting 10 μl of the 100 μl of methanol from the reconstituted sample residues, using 2 ml of plasma for analysis. In urine, the analysis of a 5-ml specimen permitted the determination of 0.020 μg of I/ml, using the same aliquot injected for GLC. However, following oral administration of single 25-mg doses of I in humans, the plasma levels fell below 0.050 μg of I/ml (Table I). In these cases, it was necessary to reconstitute the extracts in 25 μl of methanol and to inject 10-μl aliquots (Fig. 4).

Assay Specificity—Specificity of the GLC assay for I was verified by the analysis of authentic standards of two metabolites identified in dog urine. The desalkyl metabolite (II) and the 7-hydroxy metabolite (III) were both chromatographically resolved from I, with retention times of 5.8 and 11.0 min, respectively. These metabolites were not detected in the extracts of human urine following oral administration of I.

Applications to Biological Specimens—Three healthy adult male volunteers[15] received a single 25-mg tablet of I. Plasma speci-

[15] The study was conducted under the supervision of Dr. James Moore, Deer Lodge Research Unit, Deer Lodge, Mont.

mens (Subject 1) were obtained prior to drug administration and at 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 hr postadministration. Urine specimens (Subjects 2 and 3) were collected prior to drug administration from −24 to 0 hr and at the following intervals: 0–2, 2–4, 6–8, 8–12, 12–24, and 24–48 hr after drug administration.

The plasma concentrations for Subject 1 were determined for both free and total I (Table I). The free plasma levels of I are plotted in Fig. 5. The levels of conjugated I (total I − free I) measured were higher than or equal to the corresponding free I levels at each specimen time in this subject.

The urine specimens were pooled for Subject 2 and analyzed for free and total I (Table II). The rate of excretion of total I is plotted in Fig. 6 for this subject. The urine specimens of Subject 3 were analyzed only for total I, because the recovery of the dose as total I in the urines of Subjects 2 and 3 during the 48-hr interval were similar, 43.7 and 48.6%, respectively. The rate of excretion of total I for Subject 3 is plotted in Fig. 7. The levels of free I were only 5% of the dose in Subject 2, and 39% was recovered as conjugated I. The data obtained from these studies indicate that the flame-ionization GLC assay can be successfully used in the pharmacokinetic evaluation of I in humans based on both plasma level and urinary excretion data.

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[x] To whom inquiries should be directed.

NOTES

Determination of Cyanocobalamin by Thermal Decomposition of the Cyano Group Using an Ion-Selective Electrode

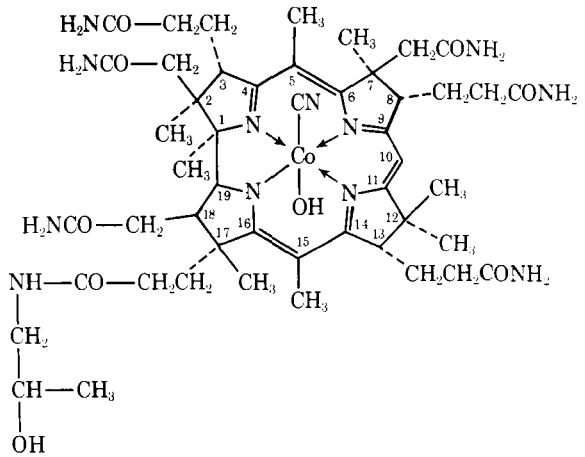
SANDU GOLDSTEIN [x] and ALEXANDRU DUCA

Abstract □ A method for the simultaneous determination of cyanocobalamin, cobinamide (Factor B), and hydroxocobalamin in the solid state is described. The method is based on heating at 120° for cobinamide and at 140–145° for cyanocobalamin (15–20 min). The cyano content in the sample is distilled as hydrocyanic acid, trapped in 0.1 *M* potassium nitrate at pH 12–13, and determined by means of the cyanide ion-selective electrode. The error of this method, statistically established, does not exceed ±3%.

Keyphrases □ Cyanocobalamin—analysis by thermal decomposition of the cyano group, solid pharmaceutical preparations □ Cobinamide—analysis by thermal decomposition of the cyano group, solid pharmaceutical preparations □ Hydroxocobalamin—analysis by thermal decomposition of the cyano group, solid pharmaceutical preparations □ Thermal decomposition—simultaneous analysis of cyanocobalamin, cobinamide, and hydroxocobalamin, solid pharmaceutical preparations □ Vitamins—cyanocobalamin, cobinamide, and hydroxocobalamin, simultaneous analysis by thermal decomposition of the cyano group, solid pharmaceutical preparations

For some time, this laboratory has been investigating methods for extracting and purifying vitamin B_{12} group substances (1–6). In recent years, these studies have been extended to the coordination chemistry of the cobalt corrinoids, the analytical chemistry of cyanocobalamin, and the thermal stability of cyanocobalamin and some of its analogs (7–11). The methods of determining vitamin B_{12} group substances have been reviewed (12–14), but no procedure was found that gives satisfactory results for the cyano group determination of cyanocobalamin in the solid state.

Therefore, it was decided to investigate the decomposition of cyanocobalamin and some analogs [cobi-



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