(43) I. J. Fidler, *Science*, 208, 1469 (1980).

(44) I. J. Fidler, S. Sore, W. E. Folger, and Z. L. Barnes, *Proc. Natl. Acad. Sci. USA*, 78, 1680 (1981).

(45) W. E. Magee, J. H. Grovenberger, and D. E. Thor, *Cancer Res.*, 38, 1173 (1979).

(46) M. B. Yatvin, J. N. Weinstein, W. H. Dennis, and R. Blumenthal, *Science*, 202, 1290 (1978).

(47) J. N. Weinstein, R. L. Magin, R. L. Cysyk, and D. S. Zaharko, *Cancer Res.*, 40, 1388 (1980).

(48) J. N. Weinstein, R. D. Klausner, T. Innerarity, E. Ralston, and R. Blumenthal, *Biochim. Biophys. Acta*, in press.

(49) M. B. Yatvin, W. Kreutz, B. A. Horwitz, and M. Shinitzky, *Science*, 210, 1253 (1980).

(50) J. L. Goldstein, R. G. W. Anderson, and M. S. Brown, *Nature (London)*, 279, 679 (1979).

(51) K. J. Widder, A. E. Senyei, and D. G. Scarpelli, *Proc. Soc. Exp.*

Biol. Med., 58, 141 (1978).

(52) H. Busch, E. Fujwara, and D. C. Firszt, *Cancer Res.*, 21, 371 (1961).

(53) S. Cohen, S. M. Beiser, and K. C. Hsu, *ibid.*, 21, 1510 (1961).

(54) K. J. Widder, G. Flouret, and A. E. Senyei, *J. Pharm. Sci.*, 68, 79 (1979).

(55) A. Senyei, K. Widder, and G. Czerlinski, *J. Appl. Phys.*, 49, 3578 (1978).

(56) K. J. Widder, A. E. Senyei, and D. F. Ranney, *Cancer Res.*, 40, 3512 (1980).

(57) A. E. Senyei, S. D. Reich, and K. J. Widder, *J. Pharm. Sci.*, 70, 389 (1981).

(58) K. J. Widder, R. M. Morris, G. Poore, D. P. Howard, and A. E. Senyei, *Proc. Natl. Acad. Sci USA*, 78, 579 (1981).

(59) R. Snyderman and M. C. Pike, *Am. J. Pathol.*, 88, 727 (1977).

RESEARCH ARTICLES

Properties, Stability, Assay, and Preliminary Pharmacokinetics of the Immunomodulatory 1,2-O-Isopropylidene-3-O-3'(N',N'-dimethylamino-n-propyl)-D-glucofuranose Hydrochloride

EDWARD R. GARRETT[x], ACHIEL VAN PEER, HELMY MAHROUS, and WALDTRAUT SCHUERMANN

Abstract □ 1,2-O-Isopropylidene-3-O-3'(N',N'-dimethylamino-n-propyl)-D-glucofuranose hydrochloride (I) is a new agent with claimed immunomodulatory action and antiviral activity. Thin-layer chromatographic procedures and identifying tests were developed to separate the drug, its synthetic precursors, and solvolytic products, and were applied to stability studies. It is stable in 0.1 N NaOH at 60° where its acid solvolysis product, 3-O-3'-(N',N'-dimethylamino-n-propyl)-D-glucose is readily degraded. The partition coefficient of I (pK_a = 9.28) between chloroform and plasma was 6.4 ± 0.2 *SEM* between pH 10.5 and 11.0. Plasma and urine (0.5 ml) adjusted to pH 11.0 were extracted with 10 ml of chloroform and the extract evaporated. The reconstituted residue in 50 μl of benzene, with the diisopropylaminoethyl analog of I as an internal standard, was derivatized with 50 μl of heptafluorobutyric anhydride at 60° for 45 min and was evaporated and reconstituted in 100 μl of benzene to be assayed for I by GLC with electron capture detection with a sensitivity of 5 ng/0.5 ml of biological fluid. The procedure was applied to

pharmacokinetics in the dog and a two-compartment body model was observed with a terminal half-life of 103–130 min. At the 40-mg dose, 60–64% was excreted renally unchanged and 20–34% as unidentified metabolities. At the 200-mg dose 82–85% was excreted renally unchanged and 15–17% as unidentified metabolites. The respective renal clearances of I were 135 and 163 ml/min. The respective total clearances of I were 204 and 191 ml/min. These metabolites were apparently unextracted with chloroform from biological fluids at pH 11 and the liquid scintillation counting (LSC) assay of extracted radiolabeled I appeared synonomous with the GLC assay of I in such fluids.
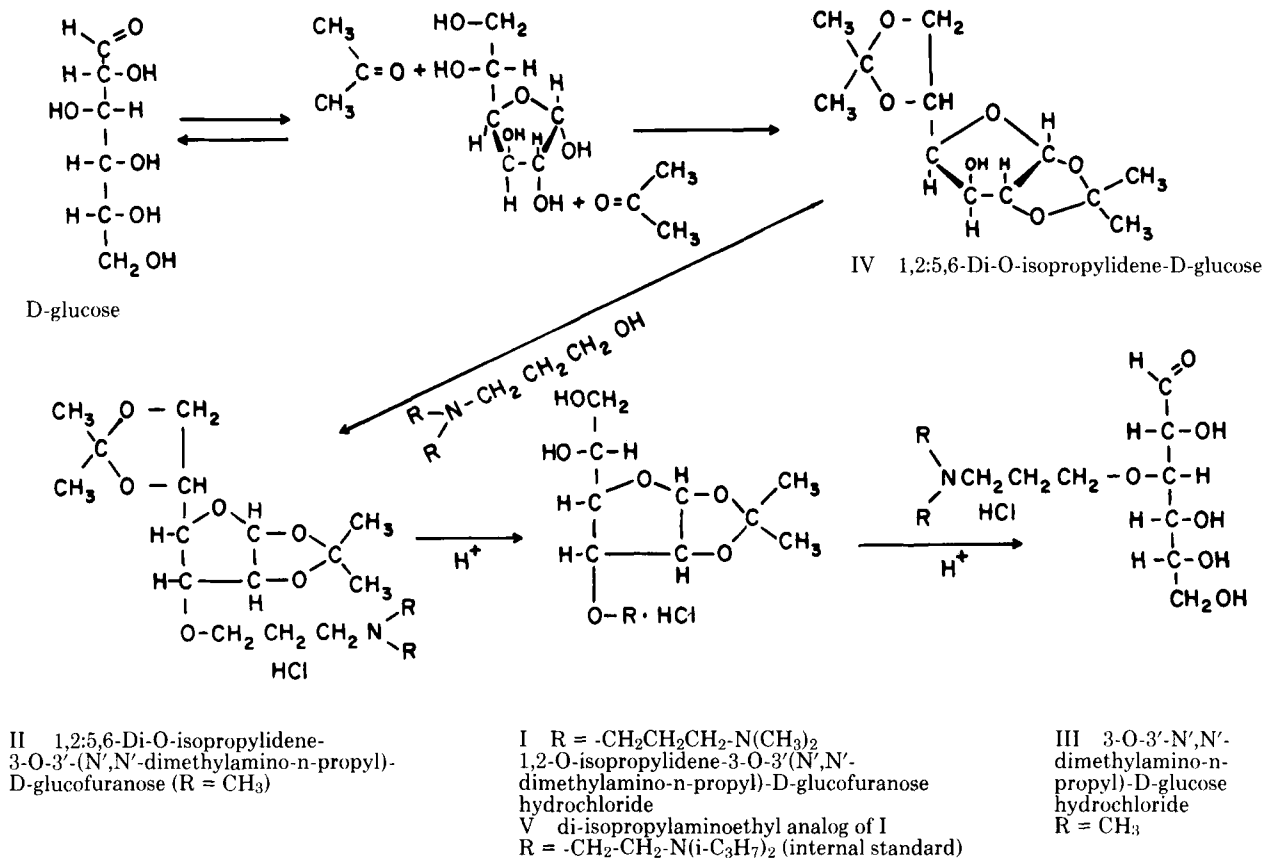
Keyphrases □ Pharmacokinetics—new immunomodulatory and antiviral agent, dogs □ GLC, electron capture—pharmacokinetics of a new immunomodulatory and antiviral agent, dogs □ Immunomodulatory agent—pharmacokinetics of a new immunomodulatory and antiviral agent, dogs

It has been shown (1–7) that 1,2-O-isopropylidene-3-O-3'-(N',N'-dimethylamino - n - propyl)-D-glucofuranose hydrochloride, I, exhibits immunomodulatory action and antiviral activity and that it possesses pro host action in which cellular immune response is augmented and macrophages are activated (8, 9). The advantageous therapeutic action is to potentiate protective responses of the immune system(6) without the inhibition of vital cell actions with their concomitant toxicities (4).

Compound I is a substituted monosaccharide of low toxicity, widely different in structure than the clinically used nonsteroidal anti-inflammatory agents with high

incidences of toxicity. An interesting argument for this possible activity of the 3-substituted monosaccharide (1–8) is that it mimics the immunological activity of the cell walls of mycobacteria with its positive cyclic guanosine monophosphate action and without its negative cyclic adenosine monophosphate effect on proliferation.

The procedure for the synthesis of I, is outlined in Scheme I. The diisopropylidene derivative of D-glucose, IV, is prepared by the addition of acetone. It is subsequently conjugated with N',N'-dimethylamino propanol, II, in the 3 position. Compound I is then prepared by selective acid hydrolysis of the 5,6-O-isopropylidene group.

D-glucose

IV   1,2:5,6-Di-O-isopropylidene-D-glucose

II   1,2:5,6-Di-O-isopropylidene-
3-O-3'-(N',N'-dimethylamino-n-propyl)-
D-glucofuranose (R = CH₃)

I   R = -CH₂CH₂CH₂-N(CH₃)₂
1,2-O-isopropylidene-3-O-3'(N',N'-
dimethylamino-n-propyl)-D-glucofuranose
hydrochloride
V   di-isopropylaminoethyl analog of I
R = -CH₂-CH₂-N(i-C₃H₇)₂ (internal standard)

III   3-O-3'-N',N'-
dimethylamino-n-
propyl)-D-glucose
hydrochloride
R = CH₃

Scheme I—Synthesis and Hydrolysis of I

Excessive acid hydrolysis could give the dimethylamino-propylglucose, III. The radiolabeled compound I was prepared with randomly labeled [¹⁴C]D-glucose.

These studies present thin-layer chromatographic (TLC) procedures to separate I from its synthetic precursors and its solvolysis products. They were applied to studies of the acid-base stabilities of these compounds. Dissociation constants and partition coefficients as functions of pH were determined. A sensitive GLC method was developed for the assay of I where the heptafluorobutyryl derivative was detected by electron capture. This procedure was applied to preliminary pharmacokinetic studies of intravenously administered I in the dog and compared to the assay of radiolabeled I in the monitored biological fluids.

EXPERIMENTAL

Materials—The following analytical grade materials were used: acetic acid[1], sodium acetate[1], sodium phosphate dibasic[1], potassium phosphate monobasic[1], sodium carbonate[1], sodium bicarbonate[1], ammonium hydroxide[1], phosphoric acid[2], sulfuric acid[1], volumetric concentrates of sodium hydroxide[3] and hydrochloric acid[3], nanograde benzene[1], n-butyl alcohol[1], chloroform suitable for GC[4], methylene chloride[1], absolute alcohol[5], ethyl acetate[4], nanograde hexane[1], isopentyl alcohol[6], methanol[4], n-propanol[2], heptafluorobutyric anhydride[7], and a silylating agent[8].

Precoated silica gel plates[9] were used for TLC separation. The following spray reagents were used: 1% ninhydrin[8] in alcohol, 4% 2,3,5-triphenyl-2H-tetrazolium chloride[10] in methanol, 3.5% p-anisidine hydrochloride[10] in n-butyl alcohol–alcohol–water (4:1:1), 3% copper acetate[2] in 18% phosphoric acid. TLC plates were also placed in tanks containing resublimed iodine[11].

1,2-O-Isopropylidene- 3 -O-3'(N'-N'-dimethylamino-n-propyl)-D-glucofuranose hydrochloride (I), the diethylaminoisopropyl analog of I, [1,2-O-isopropylidene-3-O-3'(N',N'-diethylaminoisopropyl)-D-glucofuranose hydrochloride] (VI), and the GLC internal standard diisopropylaminoethyl analog of I, [1,2-O-isopropylidene-3-O-3'(N',N'-diisopropylaminoethyl)-D-glucofuranose hydrochloride] (V) were received as hydrochlorides[12]. Diisopropylidene glucose[12], IV (mp 106°) (1,2,5,6-di-O-isopropylidene-D-glucose), monoisopropylidene glucose[12] [VII (mp 154–155°) 1,2-O-isopropylidene-D-glucose], dimethylamino-n-propyl-D-glucose hydrochloride[12] [III (mp 131–133°), 3-O-3'(N',N'-dimethylamino-n-propyl)-D-glucose hydrochloride], glucose[2], and lactose[13] were also used as standards for TLC.

Radiolabeled I, randomly ¹⁴C-labeled in the glucose component[14] (324.0 mCi/mmole), was used to prepare doses for the pharmacokinetic study. [¹⁴C]Urea[15] (55 mCi/mmole) was the internal standard for the radioactive assay. A toluene-based scintillation cocktail[16] was used in the radioactive assays.

Apparatus—A gas chromatograph[17] equipped with a ⁶³Ni-electron capture detector was used. Silylated 1.83-m glass columns were packed with 4% SE 30 and 6% OV 210 on 100/120 chromosorb W-HP[7]. The column temperature was maintained at 190°; injector temperature was 230°, and detector temperature 300°. The flow rate of the carrier gas (95%

[1] Mallinckrodt Inc., Saint Louis, MO 63147.
[2] J. T. Baker Chemical Co., Phillipsburg, N.J.
[3] Ricca Chemical Co., Arlington, TX 76012.
[4] Burdick and Jackson Laboratories, Muskegon, MI 49442.
[5] U.S. Industrial Chemicals Co., New York, NY 10016.
[6] Allied Chemical, Morristown, N.J.
[7] Supelco Inc., Bellefonte, PA 16823.
[8] Silyl 8, a silylating agent and ninhydrin, Pierce Chemical Co., Rockford, IL 61105.
[9] Silica gel 60, E. Merck Laboratories, Elmsford, NY 10523.
[10] Eastman Kodak Co., Rochester, NY 14650.
[11] Fisher Scientific Co., Fair Lawn, NJ 07410.
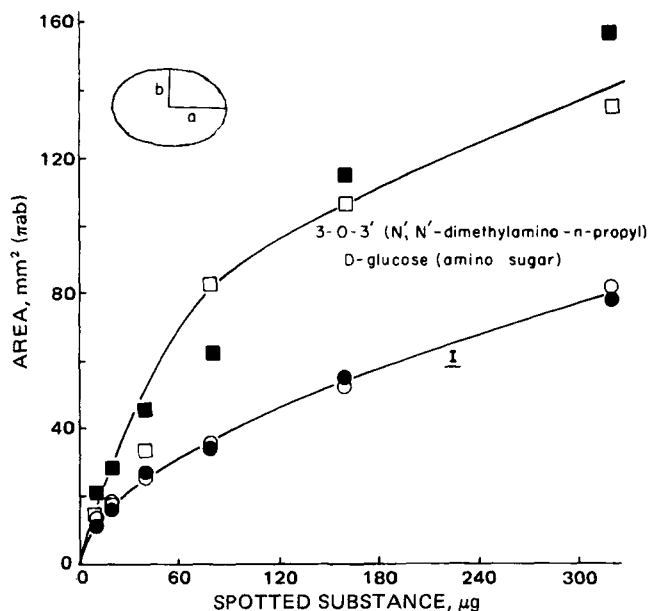[12] Greenwich Pharmaceuticals, Greenwich, CT 06830. (The I used was Lot 3646.)
[13] Strategic Medical Research Corp., Chicago, IL 60680.
[14] Lot #1141-220, New England Nuclear, Boston, MA 02118.
[15] Lot #921885, ICN Pharmaceuticals, Irvine, CA 92715.
[16] Scinti Verse, Fisher Scientific Co., Fair Lawn, NJ 07410.
[17] Model Sigma I Perkin-Elmer, Norwalk, CT 06856.

Figure 1—Quantitative TLC analyses, Area = πab versus μg, of I (O, ●), and 3-O-3′(N′,N′-dimethylamino-n-propyl)D-glucose hydrochloride, III (□, ■) as developed in n-propanol–ethyl acetate–water–NH₄OH (6:1:4:1). Key: open symbols are for the areas of the elliptical spots determined from the product of the orthogonal radii, a × b and π from ninhydrin visualization; closed symbols are from visualization after charring with 50% H₂SO₄. Various volumes of 10-μg/μl materials were spotted at the origin with successive application and drying.

Figure 2—Typical GLC chromatograms of heptafluorobutyrylated I, and the internal standard diisopropylaminoethyl analog of I, (V) in plasma and urine as well as derivatized blank urine and plasma samples, detected by electron capture. The signals are labeled as to their relative amplification for comparison. The vertical dashed lines represent the retention times of derivatized I and internal standard. The amounts and volumes of biological samples assayed are given.
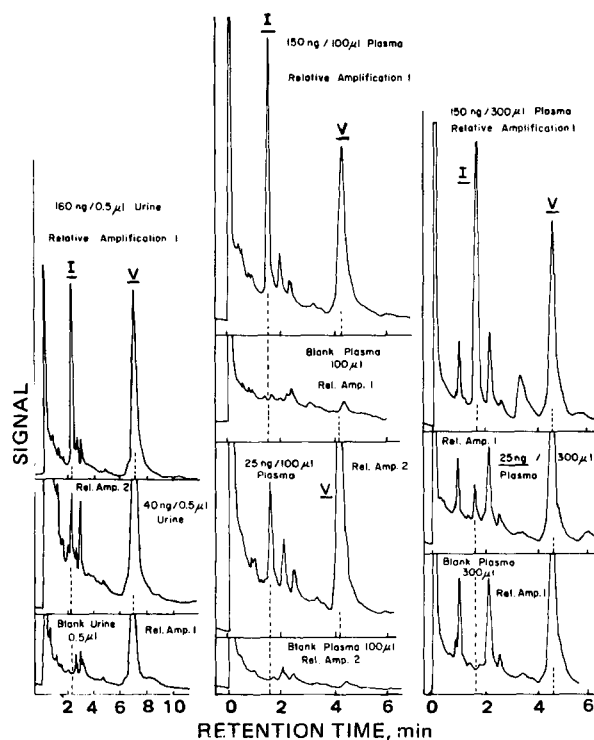
argon–5% methane) was 30 ml/min.

Radioactivity of samples was determined with a liquid scintillation counter[18].

GLC Assay of I—A solution (0.05 ml) of internal standard [10 μg diisopropylaminoethyl analog of I hydrochloride (V)/ml of distilled water] was added to an aliquot (<0.5 ml) of urine or plasma, which was diluted to 0.5 ml with distilled water. After adjustment to pH 11.0 with 0.1 N NaOH (>50 μl), the solution was extracted with 5 ml of water-saturated chloroform. The samples were shaken for 15 min and centrifuged[19] at 3000 rpm for 15 min.

The aqueous phase was removed by aspiration and 4 ml of the chloroform phase was transferred into 5-ml silylated reaction vials[7] and evaporated to dryness under a nitrogen stream[20] at room temperature. Benzene (50 μl) and heptafluorobutyric anhydride (50 μl) were added to derivatize the extracted I and the diisopropylaminoethyl analog, V, with shaking for 45 min at 60° in heating blocks[21]. The cooled reaction vials contents were evaporated (30 min) under a nitrogen stream at room temperature. The residue was reconstituted in 100 μl benzene and 1 μl was injected into the gas chromatograph.

Calibration curves were prepared using the same amounts of blank urine or plasma spiked with 0, 20, 40, 60, 80, 100, 120, 140, and 160 ng of I.

Thin-Layer Chromatography (TLC)—Glass plates coated with silica gel were used to separate the following materials (50 μg) using the system n-propanol–ethyl acetate–water–NH₄OH, 6:1:4:1. The following R_f values were obtained: diisopropylidene glucose (IV), 0.83; I (0.58); monoisopropylidene glucose (VII), 0.54; glucose, 0.32; diethylaminoisopropyl analog of I, (VI), 0.27; dimethylaminopropylglucose (III), 0.20; and lactose, 0.20. Compounds I and III could be detected by iodine vapor on the plate. Compound I could be detected as a purple spot when the plate was sprayed with 1% ninhydrin in ethanol, and VI and III gave burnt yellow colors after heating the plates 10–15 min at 110°. The compounds IV and VII, could not be detected with this reagent although the glucose spot gave a yellow coloration. Glucose compounds III and VI gave an orange spot when sprayed with 4% tetrazolium hydrochloride in methanol after heating 5 min at 110° or a brown spot when sprayed with 3.5% p-anisidine in n-butanol–ethanol–water (4:1:1) after heating 10 min at 110°,

while I gave no coloration.

All spots could be visualized with 3% copper acetate in 18% phosphoric acid after heating for 30 min at 110° or after charring with 50% sulfuric acid for 10–15 min at 120°.

Preliminary Studies of Extractability by TLC—Several organic solvents (5 ml) were used to extract 10 mg of I from 3 ml of pH 10 aqueous solution and 10 mg of III from 2 ml of pH 10 aqueous solution. The mixtures were vortexed for 30 sec and centrifuged for 5 min at 2000 rpm. Aliquots of the organic layer (100 μl) were spotted on the silica gel plates and the developed spots at the R_f of I were visualized by charring with 50% sulfuric acid spray and compared with developed spots after the application of standard I and dimethylaminopropylglucose solutions.

Hexane and hexane with 2% isopentyl alcohol extracts showed no detectable spots at the R_f of I, whereas benzene and hexane with 10 and 20% isopentyl alcohol showed small faint spots of I. The chloroform and methylene chloride extracts of I gave spots of the same size and intensity as the developed standard. Benzene extracts of I gave spots but at less intensity than with chloroform. Compound III was apparently not significantly extracted in any of these solvents as no spot was apparent from the organic extracts at the R_f of standard III.

Quantitative Analysis by TLC—Amounts (1–32 μl, 10–320 μg) of I and III were spotted on several TLC plates and developed in the system. One plate was visualized with ninhydrin (purple for I, orange for III) and one plate was visualized after spraying with 50% sulfuric acid and charring. The areas were estimated from the product of the horizontal and vertical radii times π. Plots of these areas versus spotted amounts for the two visualizations are given in Fig. 1.

Stability Estimates by TLC—Solutions (10 mg/ml) of I and III were prepared in water, 0.1 and 1.0 N NaOH, and 0.1 and 1.0 N HCl and maintained at 30 and 60°. Aliquots (20 μl) of these solutions were spotted on the TLC plates at intervals, and the developed spots visualized after spraying with ninhydrin and/or by charring with 50% sulfuric acid. The samples in alkali were neutralized with acid before spotting.

Potentiometric Titrations of I—Compound I (29 mg, 0.08486 meq) was dissolved in 20 ml water which contained 200 μl of 1.839 N NaOH and potentiometrically titrated with 1.967 N HCl with a microburette. Similar solutions without I were titrated similarly and the pH was plotted against the difference in milliliters necessary to achieve the same pH values. A typical potentiometric titration curve by this method (10) was

[18] Tricarb 460 CD, Packard Instrument Co., Downers Grove, IL 60515.
[19] International Centrifuge, International Equipment Co., Needham Heights, MA 02194.
[20] The Meyer N-Evap, Organomation Associates Inc., Shrewsbury, MA 01545.
[21] Reacti-therm Heating Modules, Pierce Chemical Co., Rockford, IL 61105.

Table I—Organic/Aqueous Partition Coefficients of 500 ng of I Between V_{org} ml of Chloroform and V_{aq} ml of Buffer or Plasma at Room Temperature

pH	V_{aq}	V_{org}	k'	k^a
			Buffer Solutions	
7.34	0.5	11.0	0.064	5.63
7.52	1.0	10.0	0.070	4.11
7.72	1.0	5.0	0.153	5.70
8.07	1.0	1.0	0.319	5.50
9.29	1.0	1.0	3.75	7.41
9.61	1.0	10.0	3.23	4.74
10.07	20.0	1.0	4.66	5.42
10.44	20.0	1.0	5.63	6.02
11.02	5.0	1.0	6.10	6.10
				Average $\overline{5.63} \pm 0.31$ SEM
			Dog Plasma	
10.10	5.0	1.0	8.4^b	
10.5	5.0	1.0	6.0	
10.5	5.0	1.0	6.5	
11.0	5.0	1.0	6.7	
11.0	5.0	1.0	6.2	
			Average $\overline{6.35} \pm 0.16$ SEM	

^{a} $k = (1 + 10^{-pH}/K'_a) k'$ where $K'_a = 5.25 \times 10^{-10}$. ^{b} Omitted from average.

obtained. The pH at half titration between the two inflection points gave pK'_a values of 9.20 and 9.28 and the apparent equivalent weight of the titrated I was 380 (theoretical, 342).

Partition Coefficient—A stock solution of the base of I was prepared in chloroform by the extraction of 0.20 ml of a sodium carbonate buffer solution (pH 10) containing 100 μg of I with 10 ml of chloroform. This 10 μg/ml solution of free base (as I equivalent) was diluted 10-fold to give a stock solution of 1.00 μg of I as the free base in 1.00 ml of chloroform.

Partition studies between appropriate volume ratios of water-saturated chloroform and buffer solutions of various pH values were effected by shaking the mixture for 15 min and then centrifuging. Higher relative volumes of the organic phase were used at the lower pH values and of the aqueous phase at higher pH values (Table I). Aliquots (0.9 of the respective volumes) of the aqueous and organic phases were taken. The organic phase was evaporated to dryness under nitrogen, derivatized, assayed by the GLC method, and the peak height ratio (PHR_{org}) to the internal standard diisopropylaminoethyl analog of I, (V) determined. The volume of aqueous phase taken was adjusted to pH 11.0 with NaOH and extracted with 10 ml chloroform and 9 ml of CHCl$_3$, taken to dryness, derivatized, assayed by the GLC method, and the peak height ratio (PHR_{aq1}) to V determined. The extracted aqueous phase was re-extracted with chloroform and the reclaimed I was assayed (PHR_{aq2}).

Preparation of the Pharmacokinetic Dose—A volume of 2.8 ml of the I-[^{14}C]glucose solution, containing 1.5 mg of I-[^{14}C]glucose hydrochloride with a labeled specific activity of 324.0 mCi/mmol, was mixed with 58.5 mg of I for the 40-mg dose, or with 298.5 mg of I for the 200-mg dose, and with 12.2 ml of sterile isotonic saline. Aliquots (10.00 ml) were injected as intravenous bolus over 20 sec into the dog. The theoretical specific activity was 8.10 mCi/mmol of I for the 40-mg dose and 1.62 mCi/mmole of the 200-mg dose.

The experimental specific activity was determined by mixing 10.00 μl of the prepared dosing solution with 10 ml of liquid scintillation fluid. The capped vials[22] were adapted to the dark for at least 6 hr before counting in the liquid scintillation counter. Experimental specific activities were 7.82 ± 0.03 (SEM) mCi/mmole and 1.554 ± 0.004 (SEM) mCi/mmole for the 40- and 200-mg dosing, respectively, and were used in the assays.

Counting Efficiencies —Counting efficiency in samples was estimated by the internal standard method. Selected samples of blank and drug-affected plasma or urine and samples of aqueous and organic phase after extraction of plasma and urine were counted before and after the addition of 22,000 dpm of internal standard [^{14}C]urea with a specific activity of 55 mCi/mmole. All capped vials[22] were dark-adapted for at least 6 hr before counting in the liquid scintillation counter. The counting efficiencies in the systems approached 100%, 99.0 ± 0.6 (SEM) % in blank and in drug-containing biological fluids. There were no significant quenching effects of 10–200 μl of dog urine or dog plasma when such amounts were added to 10 ml of liquid scintillation fluid.

Total Radioactivity—The total radioactivity of a plasma or urine sample was determined from the addition of 0.01–0.2 ml of plasma or urine to 10 ml of liquid scintillation fluid. The capped vials were dark-adapted for at least 6 hr before counting in the liquid scintillation counter.

The values (counts per minute) were converted to disintegrations per minute (dpm) by dividing by the counting efficiency after subtracting background. The total concentration of radio-labeled substances as I equivalents was calculated by dividing the dpm values by the specific activity and by taking into account the volume of plasma or urine assayed.

Determination of Radiolabeled Substances After Extraction of Plasma or Urine—Plasma (0.025–0.5 ml) or urine (0.005–0.5 ml) was transferred into a 15-ml silylated centrifuging tube. Distilled water was added to a volume of 0.5 ml, and the pH was adjusted to ~11.5 with sodium hydroxide solution (0.1 N or 1 N). The mixture was extracted with 5 ml of water-saturated chloroform by shaking for 10 min. The phases were separated by centrifugation at 3000 rpm for 10 min. An aliquot of the aqueous phase (0.3 ml) was transferred into a scintillation vial and liquid scintillation fluid (10 ml) was added. The counting efficiency of the aqueous phase was 99.0 ± 0.4 (SEM)% for plasma and 93.1 ± 0.5 (SEM)% for urine. An aliquot of the organic phase (4 ml) was evaporated in a scintillation vial under nitrogen at room temperature. Liquid scintillation fluid (10 ml) was added to the residue. The capped vials were dark-adapted and counted. The counting efficiency was calculated by the internal standard method already described. The counting efficiency was 96.7 ± 0.4 (SEM)% for the organic extract of plasma and 93.8 ± 0.8 (SEM)% for that of urine.

Concentrations, C_I^{LSC}, of I in biological fluids were calculated from liquid scintillation counting (LSC) by dividing the disintegrations per minute per milliliter of organic extract by the extraction efficiency (f_1 = 0.984) and by the experimentally determined specific activity of I (f_2), which was 50,304 dpm/μg for the 40-mg dose and 9995 dpm/μg for the 200-mg dose. Thus, when the ratio, r, of milliliters of organic phase to milliliters of biological fluid is known, C_I^{LSC} can be determined as follows:

C_I^{LSC} = concentration of I as hydrochloride in biological fluid

$$= \frac{\text{dpm/ml organic phase}}{f_1 \times f_2} \times r \quad \text{(Eq. 1)}$$

There are two methods to calculate the concentrations of radiolabeled substances in plasma or urine which cannot be assigned to I per se, presumably the metabolite concentration, C_M. This is the concentration in the aqueous phase of compounds that are not I expressed in equivalents of I as the hydrochloride after extraction of I from plasma or urine. The direct method for calculation, C_M^{direct}, is to divide the disintegrations per minute per milliliter of the aqueous phase after extraction by the specific activity of I (f_2) and to subtract the unextracted concentration of I that remained in the aqueous phase:

C_M^{direct} = apparent metabolite concentration as equivalents of the hydrochloride of I in biological sample

$$= \frac{\text{dpm/ml of extracted aqueous phase}}{f_2} - (1 - f_1)C_I^{LSC} \quad \text{(Eq. 2)}$$

[22] Kimble, Division of Owens-Illinois, Toledo, OH 43668.
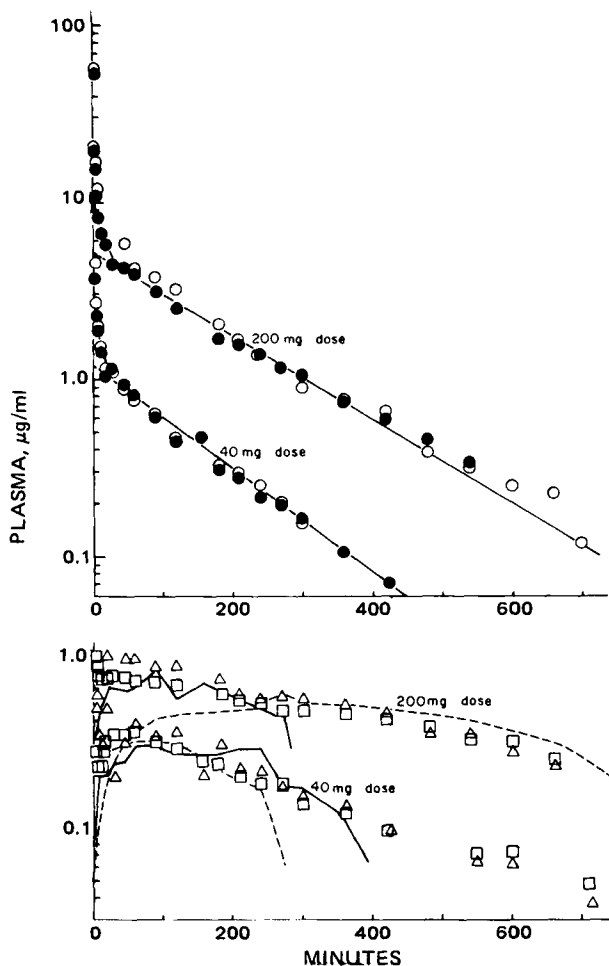
Figure 3—*Semilogarithmic plots of plasma levels of I (○, ●) and apparent total metabolite (□, △) in equivalents of I as the hydrochloride versus time for the 40- and 200-mg intravenous doses in the same dog. The symbols represent the GLC assayed I concentrations (C_T^{GLC}, ○) and the LSC assayed I concentrations (C_I^{LSC}, Eq. 1, ●) obtained from radioactivity measurement of the organic extract. The lines, $C = \Sigma A_i e^{-\alpha_i t}$, drawn through the I data were calculated from the parameters given in Table II. The plotted apparent plasma metabolite concentrations were obtained by direct LSC assay of the extracted plasma (C_M^{direct}, Eq. 2, □) and from the difference between the total plasma radioactivity count and that due to I concentrations in plasma (C_M^{diff}, Eq. 3, △). The solid lines drawn through the data were calculated apparent plasma metabolite level values based on the direct LSC assay of the previously extracted urine samples (Eq. 2) in accordance with Eq. 14 using $Cl_{met}^{tot} - Cl_x = 30.4$ and 18.5 ml/min, and $V_M = 4037$ and 3728 ml for the data of the 40- and 200-mg doses, respectively. The dashed line through the 200-mg dose data was also based on this direct LSC assay of previously extracted urine (Eq. 2) but used $Cl_{met}^{tot} - Cl_x = 27.3$ ml/min with $V_M = 16,852$ ml in Eq. 14. The dashed line through the 40-mg dose data was based on the urine assays conducted by taking the differences between total radioactivity and that due to I (Eq. 3) where $Cl_{met}^{tot} - Cl_x = 42.2$ ml/min and $V_M = 4078$ ml.*

The alternative difference method for estimating C_M^{diff} is to subtract the concentration, C_I^{LSC}, of I calculated from Eq. 1 from the apparent concentration, C_{tot}, estimated from the liquid scintillation counting of unextracted urine or plasma:

C_M^{diff} = apparent metabolite concentration as I

(hydrochloride) equivalents in biological sample

$$= \frac{\text{dpm/ml of unextracted plasma or urine}}{f_2} - C_I^{LSC} \quad \text{(Eq. 3)}$$

Pharmacokinetic Studies in the Dog—The selected dog had physiological values in the normal range: white cell count, 19,900 cells/mm³; sedimentation rate, 5 min/hr; and packed cell volume, 43%. Tests for microfilaria were negative. The pharmacokinetics of ¹⁴C-radiolabeled I in this dog were studied with intravenous bolus doses of 40- and then 200-mg after a 2-month interval.

Table II—Pharmacokinetics of Intravenous I in a 20-kg Dog

Dose (D_o), mg	40	200
Specific activity, dpm/μg	50304	9995
A^a, μg/ml	3.4	11.5
B^a	1.19	5.30
α^a, min⁻¹ ($t_{1/2}$, min)	0.195 (3.6)	0.160 (4.3)
$10^3\beta^a$	6.68 (104)	5.44 (127)
β^b	6.36 (109)	5.18 (134)
Clearances, ml/min		
$Cl_{tot}^{I\ c}$	204.4	191.2
$Cl_{ren}^{I\ d}$	135	163
$Cl_{met}^{tot\ e}$	69.4	28.2
$Cl_{met}^{tot} - Cl_x^I$	42.4ᵍ, 30.4ʰ	27.3ʰ,ⁱ, 18.5ʰ,ʲ
Cl_x^k	27ᵍ, 39ʰ	0.9ʰ,ⁱ, 9.7ʰ,ʲ
$Cl_{ren}^{M\ d}$	103.0ᵍ, 67.0ʰ	101ᵍ, 76ʰ
Apparent distribution volumes^l		
$V_C^{I\ m}$	8.7	11.9
$V_d^{I\ n}$	30.6	35.1
$V_{dextrap}^o$	33.6	37.7
$V_M^{I\ f}$	4.08ᵍ, 4.04^f	3.28ʰ,ⁱ, 18.5ʰ,ʲ
Disposition, fraction of dose^q		
ΣU_∞^I/Dose	0.60ʳ, 0.64ˢ	0.82ʳ, 0.85ˢ
ΣU_∞^M/Dose	0.34ᵍ, 0.18ʰ	0.168ᵍ, 0.145ʰ
ΣU^{tot}/Dose	0.94ᵍ,ʳ, 0.98ᵍ,ˢ	0.99ᵍ,ʳ, 1.02ᵍ,ˢ
	0.78ʰ,ʳ, 0.82ʰ,ˢ	0.97ʰ,ʳ, 1.00ʰ,ˢ

^{a} Parameters estimated from best fit of [I] (as hydrochloride) in plasma against time (Fig. 3) in accordance with $[I] = Ae^{-\alpha t} + Be^{-\beta t}$. ^{b} Estimated from Σ^- plot of $\log(\Sigma U_\infty^I - \Sigma U^I) = -\beta/2.303\,t + \log\Sigma U_\infty^I$ (Fig. 4). ^{c} Total clearance of I, Dose/AUC_∞, where AUC_∞ is the total area under I plasma level–time plot. ^{d} Renal clearance of I (or total metabolite) consistent with $\Sigma U = Cl_{ren}AUC$ (Fig. 5) where AUC is the area under I (or metabolite) plasma level–time plot for the time when the cumulative amount of I (or metabolite) in the urine, ΣU, was measured. The values for I were consistent with the renal clearance plots of Fig. 6. ^{e} The total metabolic clearance is the difference between the total and renal clearances of I, $Cl_{tot}^I - Cl_{ren}^I$. ^{f} Determined from intercept and slope of linear plots in accordance with Eq. 13, $\Sigma U^M/AUC^I = -V_M[M]/AUC^I + Cl_{met}^{tot} - Cl_x$ (Fig. 7). ^{g} From plots in Figs. 5 and 7 for ΣU^M data based on urinary amounts of metabolite calculated from the differences between total urine radioactivity count and that due I in urine (Eq. 3). ^{h} From plots in Figs. 5 and 7 for ΣU^M data based on urinary amounts of metabolite calculated from the direct LSC assay of I-extracted urine (Eq. 2). ^{i} Data from linear fitting in Fig. 7 of 200-mg dose data between 27 and 250 min. ^{j} Data from linear fitting in Fig. 7 of 200-mg dose data between 250 and 850 min. ^{k} Clearance of I by processes other than renal or the metabolic process that produced metabolites into the circulating body fluids to be subsequently renally excreted as metabolite. ^{l} Referenced to total concentrations in plasma. ^{m} Apparent volume of central compartment for I, Dose/$(A + B)$. ^{n} Apparent overall volume of distribution for I, Cl_{tot}^I/β. ^{o} Apparent extrapolated distribution volume for I on presumption of one-compartment body model, Dose/B. ^{p} Apparent overall distribution volume for metabolite. ^{q} Estimated from asymptotes of ΣU versus time plots of Fig. 5. ^{r} Based on GLC assay of urine. ^{s} Based on LSC assay of organic extract of urine (Eq. 1).

The dog was trained for several days before the experiments. He was placed on a table in a standing position and was restrained by separate straps around the forelegs and hindlegs and fixed to a horizontal bar above.

The day before the experiment the dog was weighed. Body weight was 18.6 kg at the first 40-mg dose and 20.9 kg at the 200-mg dose.

After intramuscular administration of 0.75 ml of ketamine hydrochloride[23] equivalent to 100 mg/ml ketamine, one external jugular vein of the neck was exposed and cannulated under sterile conditions with 30 cm of a catheter[24]. At least 20 cm was inserted into the vein. The incision was closed after the patency of the cannula was verified and it was filled with heparinized saline. Heparinized saline (100 units/ml) was prepared by adding 3 ml of heparin[25] (1000 units/ml) to 30 ml of bacteriostatic sodium chloride solution[26]. The dog was transferred to a metabolism cage and fasted overnight with water, *ad libitum*.

On the following day the dog was restrained on the table in the standing position. The dog was water-loaded orally 30 to 60 min before drug administration with 150 ml of tap water and was continuously infused with 0.9% NaCl solution (1 ml/min)[27] *via* a three-way stopcock[28] into the

[23] Ketaset, Veterinary Products, Bristol Lab., Syracuse, NY 13201.
[24] Intracath, intravenous placement unit, catheter size 16 GA, catheter length 12 inch, needle size 14 GA, Deseret Pharmaceutical Co., Sandy, UT 84070.
[25] Lipo-Hepin, 1000 U.S.P. units per ml (heparin sodium for injection), Riker Lab. Inc., North Ridge, CA 91324.
[26] Bacteriostatic sodium chloride inj. U.S.P., Invenex, Chagrin Falls, OH 44022.
[27] Sodium chloride injection U.S.P. McGaw Laboratories, Irvine, CA 92714.
[28] Pharmaseal Inc., Toa Alta, PR 00758.
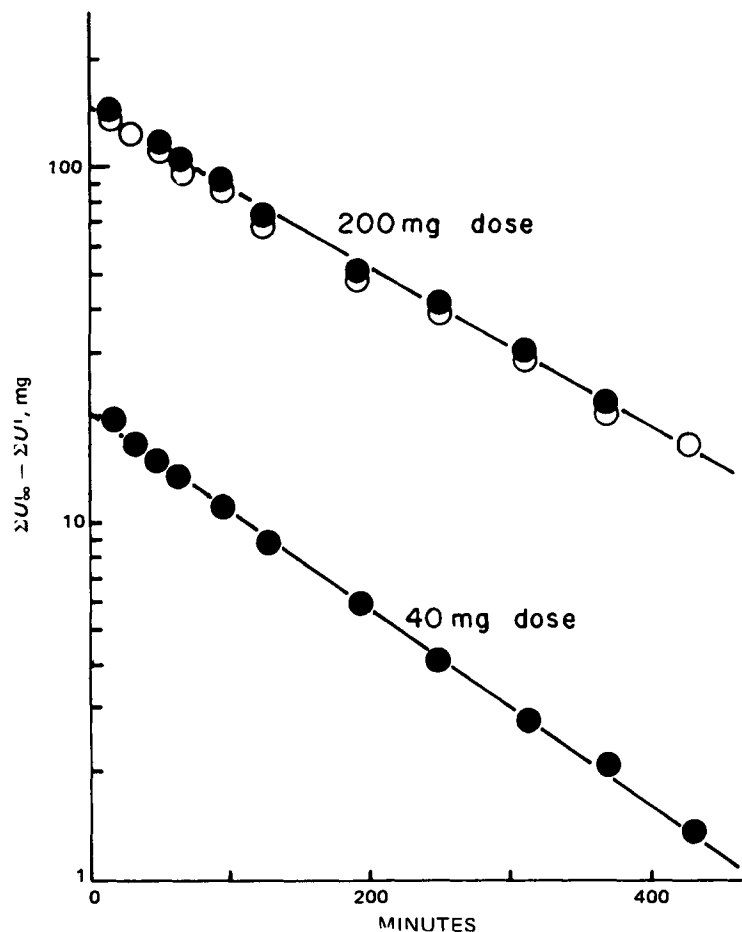
Figure 4—*Semilogarithmic plots of amounts of I not-yet-excreted versus time where ΣU_∞^I is 25.74 mg for the 40-mg dose ($\beta = 6.45 \times 10^{-3}$ min) and is 170.4 mg for the 200-mg dose ($\beta = 5.18 \times 10^{-3}$ min). Key: (O) data obtained from GLC assay of urine; (●) data from LSC assay of the organic phase of extracted urine.*

catheterized jugular vein. A transurethral catheter[29] was placed under sterile conditions to collect bladder urine.
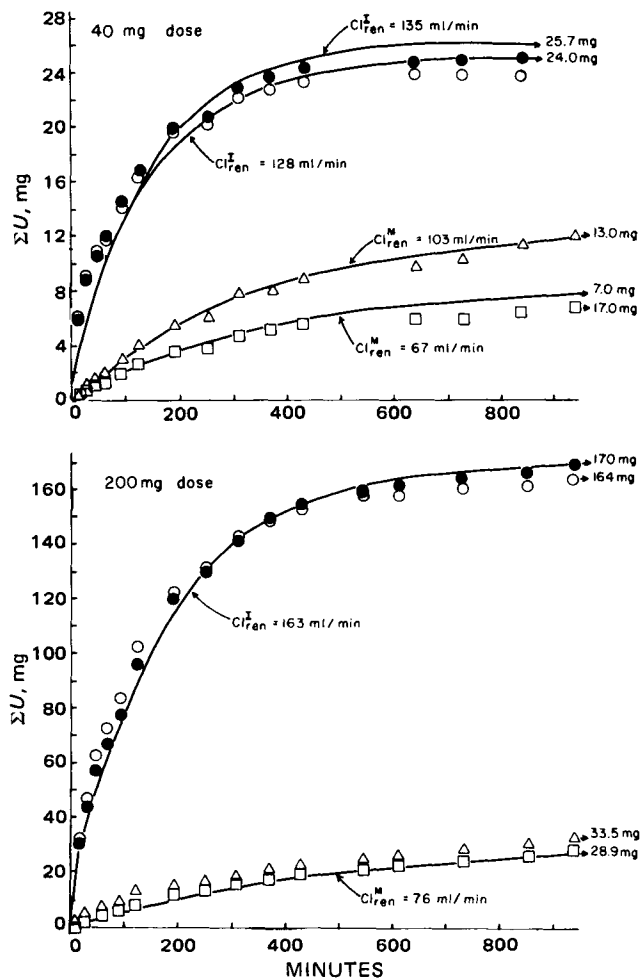
The saline drip was halted before the drug administration and a 10-ml blank blood sample was withdrawn from the jugular catheter via the three-way stopcock into a sterile, disposable syringe[30] after the dead space of the catheter (0.5 ml) had been filled with undiluted blood. The saline drip was reconnected. The blood samples taken at intervals were transferred immediately to 15-ml tubes[31] containing 143 USP units of sodium heparin and were carefully mixed and centrifuged[32] at 1500 rpm for 10 min. The plasma was transferred to a 15-ml tube[31] without additive with a Pasteur pipet and the tube was stoppered and frozen.

Urine was collected before and after the drug administration at intervals via the transurethral catheter using a sterile 20-ml syringe[30].

A ^{14}C-labeled I solution of a known amount and specific activity was injected into the jugular vein catheter over 20 sec. The dosing syringe and catheter were flushed with 10 ml of sterile saline into the jugular.

Blood was sampled at 1, 3, 5, 8, 13, 20, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, 300, 360, 420, 480, 540, 600, 660, 750, 840, 1360, 1590, 1800, 2040, 2270, and 2840 min. Adequate volumes of blood were withdrawn: 2 ml for the first 30 min samples, 3 ml for samples between 30 and 150 min, 4 ml for samples between 150 and 300 min, and 5 ml for all subsequent samples. Hematocrits were determined using a microhematocrit centrifuge and reader[33] on selected blood samples prior to centrifugation. An aliquot of plasma was removed to measure total carbon 14. The remaining plasma was stored in the freezer.

Urine was collected from the transurethral catheter at approximately 15, 30, 45, 60, 90, 120, 190, 250, 310, 370, 430, 490, 540, 610, 730, 850, 1380,

[29] Urine catheter, 8FR, 16 inch long, Davol Inc., Providence, RI 02901.
[30] Monoject, St. Louis, MO 63103.
[31] Vacutainer tubes, Becton-Dickinson and Co., Rutherford, NJ 07070.
[32] Safeguard Centrifuge, Clay Adams Co., Inc., New York, N.Y.
[33] IEC MB Centrifuge, Damon/IEC Division, Needham Heights, MA 02194.

Figure 5—*Plots of the experimental values of cumulative I, ΣU^I, and the total metabolite, ΣU^M, in urine against time. The curves through the data points are the theoretical values calculated from $\Sigma U^I = Cl_{ren}^I AUC^I$ and $\Sigma U^M = Cl_{ren}^M AUC^M$ for the labeled renal clearances of I, Cl_{ren}^I, and total metabolite, Cl_{ren}^M, is plasma where AUC^I and AUC^M are their respective areas under plasma level–time curves at the time when the cumulative amounts in the urine, ΣU, were measured. Key: (O) data obtained by GLC assay of I in urine; (●) data from LSC assay of I in the organic phase of extracted urine (Eq. 1). The total metabolites excreted in urine were assayed by the direct LSC assay of I-extracted urine (Eq. 2, ⊡) and from the difference between the total urine radioactivity count and that due to I in urine (Eq. 3, △).*

1590, 1800, 2040, 2280, and 2800 min. The volume and pH of each thoroughly mixed urine collect were measured immediately after the withdrawal from the catheter. An aliquot of the fresh urine was transferred to a 15-ml tube and was diluted in a ratio of 1:10, 1:20, 1:50, or 1:100 with distilled water, dependent on the sample time and the volume of each urine collected. Appropriate volumes of undiluted and diluted urine were removed to determine total carbon 14. The remaining urine collections were divided into 10-ml portions and stored in the freezer.

The dog was returned to the metabolism cage 14 hr after the drug administration.

RESULTS AND DISCUSSION

Properties of I—Compound I as the hydrochloride is a white crystalline solid, mp 181–183°, mw 341.74, mass spectrum: m/z 305 (m+).

Anal—Calc % for $C_{14}H_{28}O_6ClN$: C = 49.19; H, 8.19; N, 4.09; Cl, 10.39; O, 28.11. Found: C, 49.31; H, 8.38; N, 3.06; Cl, 10.50; O, 27.9%.

It is highly soluble in water and soluble in methanol and hot ethanol. It is recrystallized from methanol. The free amine from aqueous solutions of I buffered at pH 10 was determined by TLC of the organic extracts to be highly extracted in chloroform and methylene chloride, less efficiently extracted in benzene, and relatively nonextracted in hexane; although, hexane plus 10 and 20% isopentyl alcohol showed small faint spots of I. The dimethylaminopropylglucose, III, was not significantly extracted

by any of these solvents. Compound I has an apparent pK'_a of 9.28 by potentiometric titration, and the optical rotation of a 10% aqueous solution at pH 7 is $-24.5 \pm 0.5°$ and an index of refraction of $n_D^{23°} = 1.3465$. The optical rotation of III is $+ 37.0 \pm 0.5°$. The infrared spectrum of I (KBr disc) shows the presence of a split OH-band in the 3400 cm^{-1} region and characteristic bands in 1000–1400 cm^{-1} for isopropylidene groups. It cannot be readily differentiated from II and IV, although IV can be removed from acid solutions of I by chloroform extraction.

The compounds I, III, IV, and V can be separated from each other by TLC by the system n-propanol–ethyl acetate–water–NH$_4$OH, 6:1:4:1.

Stability Estimates by TLC—Compound I gave the same spot with the same developed color intensity as the standard on being subjected to 0.1 and 1.0 N NaOH at both 30 and 60° for 3, 24, 50, and 400 hr. The dimethylaminopropylglucose, III, $R_f = 0.20$, produced two additional spots at R_f values of 0.13 and 0.37 on being subjected to 0.1 N NaOH at 30° for 3 hr whereas, in 1.0 N NaOH, most of the amino sugar spot of 0.20 had been transformed to that at $R_f = 0.37$ at 3 hr. The developed spot assigned to compound III had disappeared at 24 hr in both 0.1 and 1.0 N NaOH at 30°.

Compound III gave the same spot with the same developed color intensity as the standard on being subjected to 0.1 and 1.0 N HCl at both 30 and 60° for 3, 24, 50, and 400 hr. Compound I ($R_f = 0.52$) produced a considerable quantity of the open chain dimethylaminopropylglucose (III) in 1 hr at 30° in 0.1 N HCl and was completely transformed to III in 1 hr at 60 and 30° in 1.0 N HCl.

Thus, compound I has high stability in 0.1 and 1 N NaOH, whereas III is rapidly degraded. I is rapidly degraded in 0.1 and 1 N HCl to stable III by splitting of the ether linkages to liberate acetone. The alkaline degradation products of III showed compounds with high UV spectrophotometric absorbances at <550 nm whereas undegraded III had no absorbance down to 210 nm.

Partition Coefficients and Extraction Efficiency of I from Aqueous Solutions and Plasma by Chloroform—The apparent partition coefficients, k', was calculated for chloroform extraction of the aqueous phase at a given pH value by:

$$k' = \frac{[I]_{org}}{[I]_{aq}} = \frac{(PHR)_{org} \times 1/\gamma_1}{[(PHR)_{aq_1} + (PHR)_{aq_2}] \times 1/\gamma_2 \times 1/\gamma_3} \times \frac{V_{aq}}{V_{org}} \quad (Eq.\ 4)$$

where the peak height ratios determined for I relative to V were PHR$_{org}$ for the assay of a γ_1 fraction of the volume of the organic phase, and PHR$_{aq}$ for the assay of a γ_3 fraction of the volume of chloroform used to extract a γ_2 fraction of the volume of the aqueous phase. The PHR$_{aq_1}$ and PHR$_{aq_2}$ were the peak height ratios for the first and second extractions of the previously equilibrated aqueous phase. These two extractions accounted for practically all of the I partitioned into the buffer or plasma when a volume of V_{aq} milliliters of buffer or plasma were initially equilibrated with V_{org} milliliters of chloroform (Table I).

This apparent partition coefficient can be defined as:

$$k' = \frac{[I]_{org}}{[I]_{aq}} = \frac{[I\ as\ base]_{org}}{[I\ as\ base]_{aq} + [protonated\ I]_{aq}} \quad (Eq.\ 5)$$

Since:

$$1/k' = \frac{[I\ as\ base]_{aq}}{[I\ as\ base]_{org}}$$

$$+ \frac{[protonated\ I]_{aq}}{[I\ as\ base]_{org}} = 1/k + \frac{[protonated\ I]_{aq}}{[I\ as\ base]_{org}} \quad (Eq.\ 6)$$

where k is the intrinsic partition coefficient for the free base of I between the organic and aqueous phases, and:

$$[protonated\ I]_{aq} = [I\ as\ base]_{aq}\ [H^+]/K'_a \quad (Eq.\ 7)$$

where $K'_a = 5.25 \times 10^{-10}$ is the apparent dissociation constant of I ($pK'_a = 9.28$) and $[H^+] = 10^{-pH}$ is the activity of the hydrogen ion in the extracted buffer solution or plasma. Then by substitution of Eq. 7 into Eq. 6,

$$1/k' = 1/k + \frac{[I\ as\ base]_{aq}\ [H^+]}{[I\ as\ base]_{org}\ K'_a} = 1/k + \frac{[H^+]}{kK'_a} \quad (Eq.\ 8)$$

It follows that the intrinsic partition coefficient is

$$k = (1 + [H^+]/K'_a)k' \quad (Eq.\ 9)$$

and these calculated values are given in Table I.

The standard assay of I in plasma or urine extracts 0.5 ml with 5 ml of chloroform at pH 11.5. Thus, for partition coefficients of 5.63–6.35, 98.4% of I is extracted into the organic phase.

GLC Assay of I—Typical chromatograms of I, and the internal
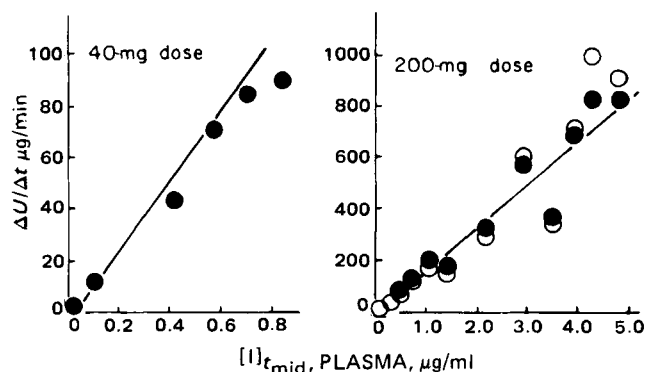
Figure 6—Renal clearance plots of μg/min of I excreted into the urine versus the plasma levels of I, $[I]_{t_{mid}}$ at the mid-time of the collected interval. Key: (○) data obtained by GLC assay of urine; (●) data from LSC assay of the organic phase of extracted urine (Eq. 1). The lines drawn through the plots are for renal clearances of 135 and 163 ml/min for the 40- and 200-mg I doses, respectively.

standard diisopropylaminoethyl I are given in Fig. 2 and, for the specified conditions, had retention times of 1.6 and 4.3 min, respectively. The limit of detection was 5–10 ng of I. The regression analysis of the assayed amounts of I in 0.5 ml of plasma or urine against the PHR were effected in accordance with:

$$C \pm s_{C\ PHR} = (m \pm s_m)PHR + (b \pm s_b) \quad (Eq.\ 10)$$

where C is the concentration of I in 0.5 ml of aqueous fluid, PHR is the peak height ratio of I to the internal standard (500 ng of diisopropylaminoethyl analog of I in 0.5 ml of the extracted aqueous phase), m is the slope, b is the intercept, $s_{C\ PHR}$ is the standard error of estimate of concentration/0.5 ml on the PHR, s_m is the standard error of the regression coefficient m, and s_b is the standard error of the intercept b.

The intercepts of such calibration curves were not significantly different from zero. The standard errors of estimates of the concentrations from these calibration curves ranged between 4.9 and 6.3 ng/0.5 ml of assayed fluid.

A typical calibration curve for GLC–electron capture detection of derivatized I with 500 ng of the diisopropylaminoethyl analog of I (V), as the internal standard gave the following regression equation for amounts (A) in 0.5 μl of urine:

$$A \pm 6.34\ ng = (144.6 \pm 7.1)\ PHR - 4.6 \pm 5.2 \quad (Eq.\ 11)$$

For amounts in 100 μl of plasma,

$$A \pm 4.87\ ng = (162.0 \pm 6.1)\ PHR - 10.0 \pm 4.2 \quad (Eq.\ 12)$$

Comparison of GLC and LSC assays—When radiolabeled I was extracted from 0.5 ml of water, plasma, or urine that had been adjusted to pH 11.5 with 5 ml of chloroform, reextraction of the separated aqueous phase with the same amount of chloroform showed less than 2% of the original radioactivity in the second extract. This was consistent with the 1.6% that had been estimated from the measured partition coefficients to be nonextracted under these conditions. The paired assay values of I by GLC and LSC, were highly correlated with a correlation coefficient of $r = 0.995$ for the 40-mg dose for plasma values between 0.15 and 2.6 μg of I/ml of plasma. The paired values were challenged by the Student t test ($t = 1.12; df = 13$) and the null hypothesis that the values were the same from both assays could not be rejected. Similarly, the correlation coefficient was 0.991 for the 200-mg dose for plasma values between 0.3 and 4.0 μg I/ml of plasma. The null hypothesis of no difference between the LSC and GLC assays of I in plasma could not be rejected ($t = 1.73; df = 9$). The correlation can be observed from Fig. 3.

The correlation coefficients for the paired GLC and LSC urine assays of I were 0.995 and 0.986 at the 40- and 200-mg doses, respectively. The t values for the 40-mg dose were 0.16, $df = 6$, and 1.85, $df = 12$, for the amounts in the urine fractions collected in the interval 0–193 min and 0–732 min, respectively. The t values for the 200-mg dose were 0.25, $df = 11$ and 0.60, $df = 16$, for the amounts in the urine fractions collected between 0–546 min and 0–747 min, respectively.

Thus, it can be concluded that the LSC assay of the radiolabeled I in the chloroform extract of plasma and urine of dogs administered ^{14}C-labeled I intravenously does not give significantly different results than the GLC assay of the extract. Thus, any radiolabeled metabolites that
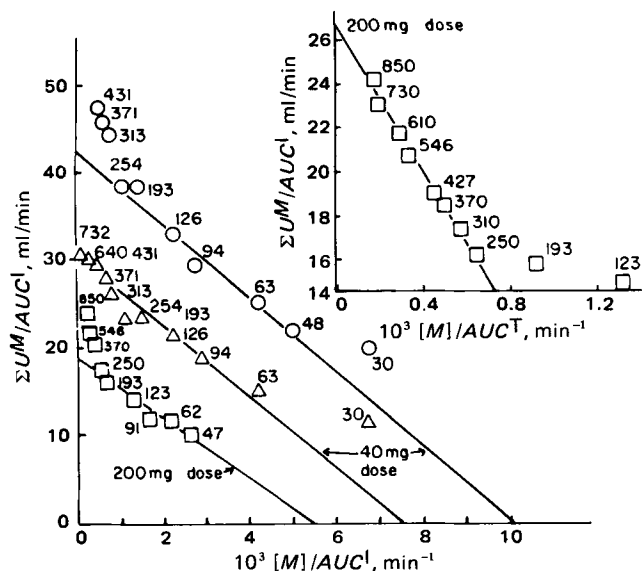
Figure 7—*Plots in accordance with Eq. 13:* $\Sigma U^M/AUC^I = -V_M[M]/AUC^I + Cl_{met}^{tot} - Cl_x$ *where the intercepts of* $Cl_{met}^{tot} - Cl_x$ *and the negative slopes of* V_M, *respectively, were for the 40-mg dose; 42.4 ml/min and 4078 ml when the urinary amounts of metabolite,* U^M, *were calculated from the difference between total urine radioactivity count and that due I in urine (Eq. 3,* ⊙*); 30.4 ml/min and 4037 ml when the urinary amounts of metabolite,* U^M, *were calculated from the direct LSC assay of I-extracted urine (Eq. 2,* △*). The respective values for the 200-mg dose when the urinary amounts of metabolite,* U^M, *were calculated from the direct LSC assay of I-extracted urine (Eq. 2,* ▫*) were 27.3 ml/min and 3278 ml when the data between 47 and 250 min were fitted linearly and 18.5 ml/min and 16,850 ml when the data between 250 and 850 min were fitted linearly (see insert).*

may exist in the plasma or urine are not readily or significantly extracted into chloroform under the conditions used.

Preliminary Pharmacokinetics of I in the Dog—The pharmacokinetic parameters are listed in Table II. Details of their calculations and the definitions of the symbols have been given in other publications (11, 12). These preliminary studies indicate that the plasma level–time curve of intravenously administered I in the dog conforms to a two-compartment body model (Fig. 3) which can be characterized by the sum of two exponentials. The respective half-lives of the two phases are 3.6–4.3 and 104–127 min. The respective estimated terminal half-lives from the urine sigma minus plot (Fig. 4) and the plasma level–time curves (Fig. 3) were the same (Table II). These limited studies do not permit the definitive conclusion that I may have dose dependent kinetics and a potentially saturable elimination process even though the higher dose had a longer half-life.

The apparent distribution volumes of I, in reference to total concentration in plasma for the central compartment (V_C) and equilibrated tissues (V_d), were 8.7–11.9 and 31–35 liters, respectively.

Clearances—The total clearances of I were similar for both the 40- and 200-mg doses, 204 and 191 ml/min (Table II). The renal clearances for these respective doses were 135 and 163 ml/min (Figs. 5 and 6) indicative of excess tubular secretion in addition to glomerular filtration, since a 12-kg dog has an inulin (glomerular filtration) clearance of 51 ± 12 ml/min (13). The possibility of saturable tubular reabsorption could explain the apparent variation of renal clearance with dose if it were substantiated by further studies.

The extra-renal clearances, Cl_{met}^{tot}, are thus estimable from the differences between the total and renal clearances and are 69.4 and 28.2 ml/min at the 40- and 200-mg doses, respectively, and do indicate a possible saturable metabolism with dose-dependent pharmacokinetics.

Urinary Recoveries—The urinary recovery of unchanged I was 60–64 and 82–85% at the 40- and 200-mg doses, respectively. This was consistent with the fact of higher renal clearance of I at the higher dose. Whereas the calculated total amounts of radiolabeled metabolites excreted in the urine at the higher 200-mg dose, *i.e.*, radiolabeled amounts not extractable into chloroform at pH 11.5, were reasonably the same when calculated from both the direct assay of extracted urine 15% (Eq. 2); and the differences between total urine radioactivity and that due I 17% (Eq. 3). This was not true for the 40-mg dose. The respective values at this dose were 18 and 34%. The only explanation other than technological for this

discrepancy would be that some radiolabeled metabolite was extracted into the organic phase in sufficiently small amounts so that statistics of identity between the GLC and LSC analyses of I in this phase were not significantly perturbed.

The total urine recovery of I and its metabolite was 100 ± 2% at the 200-mg dose. It was 96 ± 2 and 80 ± 2% for the 40-mg dose when the difference (Eq. 2) and direct (Eq. 3) assays for metabolites were used, respectively.

Attempted Estimates of Pharmacokinetic Properties of Metabolites—If only one metabolite, M, were produced from I, and if this metabolite in the systemic circulation were only renally excreted, the following equation would be valid for constant clearances (12):

$$\Sigma U^M/AUC^I = -V_M[M]/AUC^I + Cl_{met}^{tot} - Cl_x \qquad (Eq.\ 13)$$

where [M] and ΣU^M are the respective plasma concentrations of metabolite and the cumulative amount of metabolite excreted into the urine at time, t, corresponding to the area under the plasma level-time curve of I, AUC^I, to that time; where V_M is the estimated overall apparent distribution volume of the metabolite; and where $C_{met}^{tot} - Cl_x$ is the clearance of I to systematically circulating metabolite.

The V_M and $Cl_{met}^{tot} - Cl_x$ values can be estimated from the slope and intercept, respectively, of plots of the quotients in Eq. 13 (Fig. 7). The discrepancy in the estimates of ΣU^M by the direct and difference methods of radiolabeled metabolites in urine for the 40-mg dose gave different values for $Cl_{met}^{tot} - Cl_x$ of 30.4 and 42.4 ml/min, respectively; even though the apparent distribution volumes, V_M, for the metabolite were the same, 4.0 liters. As a consequence, the residual clearances of I, $Cl_x = Cl_{met}^{tot} - (Cl_{met}^{tot} - Cl_x)$, which could be due to metabolite partitioning or I excretion into the bile, differed also (Table II). The estimated values of these parameters for the 200-mg dose depended on where the data were presumed to be linear (Fig. 7). The assumption of validity for the early time data (<250 min) gave a V_M value of 3.3 liters nearest that obtained for the same time range of data for the 40-mg dose. The assumption of validity of the terminal data (>250 min) in Fig. 7 gave an extremely high apparent volume of distribution for the metabolite, a V_M of 18.5 liters.

Rearrangement of Eq. 13 and use of these estimated parameters permit the estimation of theoretical plasma levels of a possible metabolite in accordance with:

$$[M]_{calc} = \frac{(Cl_{met}^{tot} - Cl_x)AUC_I - \Sigma U^M}{V_M} \qquad (Eq.\ 14)$$

and the lines drawn through the experimental plasma concentrations of apparent metabolite were calculated on these premises (Fig. 3). The fact that the parameters chosen from Fig. 7 for the early time intervals gave reasonable fits for the early time, but not the latter (also compare the two fits of metabolite plasma levels at the 200-mg dose), is a strong indication that either the simple hypothesis of a single metabolite being formed from I is invalid or that the formed metabolite is partitioned into bile and undergoes an enterohepatic recirculation which elevates its terminal plasma levels above the theoretical value calculated from Eq. 14.

REFERENCES

(1) P. Gordon, B. Ronson, and S. V. Kulkarni, *Am. Soc. Microbiol.*, **74**, 165 (1973).

(2) P. Gordon, B. Ronson, and D. P. Mucha, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, **32**, 807 (1975).

(3) P. Gordon, B. Ronson, and D. P. Mucha, *Am. Soc. Microbiol.*, **75**, 6 (1975).

(4) J. A. Majde and P. Gordon, "Immunomodulation by 1,2,-*O*-Isopropylidene-3-*O*-3'-(*N'*,*N'*-dimethylamino-*n*-propyl)-D-glucofuranose SM-1213, a Drug with Antiviral Activity." Presented at the 16th Interscience Conference on Antimicrobial Agents and Chemotherapy, Abstract, Chicago, Ill. (1976).

(5) J. A. Majde and P. Gordon "Maintenance of Immuno Responsiveness During Subclinical Herpes Virus Infection in Mice by SM-1213, a Drug with Antiviral Activity." Presented at the 17th Interscience Conference on Antimicrobial Agents and Chemotherapy, New York, N.Y. (1977).

(6) P. Gordon, T. Hashimoto, and J. A. Majde, "*In Vitro* and *In Vivo* Enhancement of Leukocyte Functions by 1,2-*O*-Isopropylidene-3-*O*-3'-(*N'*,*N'*-dimethylamino-*n*-propyl)-D-glucofuranose (SM 1213), a Microbicidal Immunomodulator." Presented at the 18th Interscience Conference on Antimicrobial Agents and Chemotherapy, Atlanta, Ga. (1978).

(7) P. Gordon and H. Shinhai, *Am. Soc. Microbiol.*, **79**, 14 (1979).

(8) J. W. Hadden, *Cancer Treat. Rep.*, **62**, 1981 (1978).
(9) J. W. Hadden, A. England, J. R. Sadlik, and E. M. Hadden, *Int. J. Immunopharmacol.*, **1**, 17 (1979).
(10) T. V. Parke and W. W. Davis, *Anal. Chem.*, **26**, 642 (1954).
(11) E. R. Garrett and A. J. Jackson, *J. Pharm. Sci.*, **68**, 753 (1979).
(12) E. R. Garrett and T. Gürkan, *ibid.*, **69**, 1116 (1980).
(13) H. M. Smith, "Principles of Renal Physiology," Oxford University Press, New York, N.Y., 1956, p. 32.

Systematic Error Associated with Apparatus 2 of the USP Dissolution Test II: Effects of Deviations in Vessel Curvature from That of a Sphere

DON C. COX*×, CLYDE E. WELLS*, WILLIAM B. FURMAN*, THOMAS S. SAVAGE‡, and ALFRED C. KING§

Received April 16, 1981, from the *Food and Drug Administration: *National Center for Drug Analysis, St. Louis, MO 63101; ‡Los Angeles, CA 90015; and §Winchester Engineering and Analytical Center, Winchester, MA 01890.* Accepted for publication July 23, 1981.

Abstract □ Dissolution vessels made from glass or plastic are recognized by the USP as being suitable for dissolution testing. Glass vessels with a bottom inside curvature flatter than that of a sphere can cause a high bias in dissolution results; vessels with a steeper curvature can cause a low bias. The inside bottom curvature of plastic vessels adhered closely to the curvature of a sphere. The plastic vessels are preferable for use if the drug is not adsorbed and the vessel is not attacked by the dissolution medium. Bias in results between individual positions of a dissolution apparatus was traced to two shafts which were not vertical.

Keyphrases □ Dissolution—systematic error associated with USP dissolution Apparatus 2 □ USP—error associated with dissolution Apparatus 2 □ Apparatus—systematic error associated with USP dissolution Apparatus 2

The effect of variations in physical dimensions of the vessels on dissolution results was studied. The study of the molded glass vessels was conducted under the test conditions described in the Fourth Supplement to USP XIX (3); *i.e.*, the stirring element consisted of a shaft with a detachable paddle blade positioned on the side of the shaft. The tubing-produced glass vessels and plastic vessels were studied with the currently official stirring element (4). The tubing-produced glass vessels and plastic vessels are compared for their suitability for use in the USP dissolution test for prednisone tablets.

EXPERIMENTAL

A commercial sample of 5-mg prednisone tablets was used to check the performance of dissolution equipment. Dissolution results from these tablets were reported recently (5). The tablets are reasonably uniform in total drug content. A randomly selected 60-tablet subsample gave an overall average result of 97.2% of label claim with a coefficient of variation (CV) of 2.88% in a content-uniformity assay (6). The average weight of the tablets was 143 mg. This tablet sample was referred to as Tablet 1 (7).

In 1979 a commercial sample of 10-mg prednisone tablets, referred to as Tablet 2, was characterized (the supply of Tablet 1 was running low). This second performance standard gave an average result of 100.0% of label claim with a CV of 1.5% when 20 tablets were subjected to content-uniformity assay. The average tablet weight was 225 mg. Both Tablet 1 and Tablet 2 disintegrate within 2 min into coarse, insoluble granules which stay on the bottom of the vessel throughout the test. The granules from three disintegrated units of Tablet 1 visually appear to occupy about the same volume in the bottom of the vessel as the granules from one disintegrated unit of Tablet 2.

Evaluation of Molded Glass Vessels—Three laboratories[3], each using a commercially available six-spindle dissolution apparatus[4], compared dissolution results from four sets of six molded glass vessels. Tablet 1 was used by all three laboratories. The data from this experiment were collected prior to the modification of the apparatus; *i.e.*, the apparatus described in the Fourth Supplement to USP XIX was used. All other experimental data reported in this paper were collected using the current apparatus.

Evaluation of Glass Tubing-Produced Vessels—An apparatus described previously (7) was selected for this experiment. The apparatus

Only one manufacturer[1] produces a glass, round-bottom vessel (1) suitable for the multiple-spindle drive equipment used for the USP dissolution test (2). The manufacturing process was changed in 1978 in an attempt to improve the vessel. The vessel was formed manually in the older process by using a mold to which the outside of the vessel could conform. These molded vessels vary considerably with respect to weight, inside cylindrical diameter, and inside bottom curvature. In the newer process the vessel is made from large-bore glass tubing, and the bottom of the vessel is shaped manually from the outside. The tubing-produced vessels examined compare closely with respect to weight and inside cylindrical diameter. However, the inside bottom curvature varies from one vessel to the next. Vessels made from the tubing process are in widespread use. Many vessels made from the older process do not pass the USP requirement that the inside diameter be 10.0–10.5 cm.

A plastic vessel[2] formed by injection molding has been available since 1979. The variation in physical dimensions (including bottom curvature) of individual plastic vessels is less than that of glass vessels because of the manner in which they are produced. Both types of vessels are currently recognized by the USP as being suitable for dissolution testing.

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[1] Kimble, Division of Owens-Illinois, Inc., Vineland, NJ 08360.
[2] Eli Lilly and Co., Indianapolis, IN 46206.

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[3] Food and Drug Administration laboratories located in Los Angeles, Calif., St. Louis, Mo., and Winchester, Mass.
[4] Hanson Research Corp., Northridge, CA 91324.