Inulin Clearance in Mice as a Standard for Radiopharmaceutical Bioassay (35044)

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To use radiopharmaceutical compounds diagnostically, it is of maximum importance to gain assurance that the compounds have not been altered before or after administration, and that they do not alter the excretory pattern as a result of intimate radiation exposure of renal cells by the isotope selected. As an initial step in gaining such assurance, it appeared that utilization of the known stability of glomerular filtration rates might be of value. A procedure, described below, was devised to determine inulin clearance in mice which, if the data were sufficiently reproducible, could be used as one standard parameter to test radiopharmaceuticals primarily excreted by renal mechanisms.

Materials. 1. ^{14}C-Iunlin–carboxyl (New England Nuclear Corporation), specific activity: 3.96 mCi/g. The injection solution contains 0.05 μCi/12.7 μg per 0.01 ml.

2. Yale–Swiss mice, male, 5 weeks old, averaging 20 g (range 19–21 g).

3. Packard Model 3320 Tri-Carb Spectrometer was used for sample counting.

4. Scintillation fluid for: (A) *Blood samples*: (For 1 liter mix) (1): 0.133 g POPOP (1,4-bis[2-(5-phenyloxazolyl]-benzene); 6.3 g PPO(2,5-diphenyloxazole); 44.5 g naphthalene; 712.0 ml dioxane; 178.0 ml tolune; 27.0 ml ethanol (anhydrous); 32.0 g Cab-O-sil (Packard Instrument Co.).

(B) Urine samples. (for 1 liter mix) 0.375 g POPOP; 7.0 g PPO; 150.0 g naphthalene; 843 ml dioxane.

5. Mouse sacrifice unit (a plastic box contains a sheet-nickel floor as one electrode and a series of needles protruding from a movable plastic cover as the other electrode; this makes possible almost instant painless sacrifice using standard laboratory outlets, 110–120 V).

6. A modification of the clearance procedure of Farr and Konikowski (2) was followed.

Procedure. Each mouse was induced to urinate before injection of the inulin solution by the following manual maneuver. The mouse was caught by pinning the tail between the fifth finger and the hypothenar eminence. The ears were then rapidly fixed between the thumb and index finger and firm, continuing pressure was applied to the back by the dorsum of the middle finger; held in this position, the mouse will promptly empty its bladder, as previously verified by autopsy examination. For excretion time intervals of 60 min or less, a cotton surgical suture, No. 00, was used to ligate the penis immediately after the above maneuver. This ligation procedure is surprisingly well tolerated. Maximum duration for penis ligation was 70 min for, as was previously shown (2) with ligation after this period of time, the bladder shows a diminution rather than a further increase in the excreted compound. For the collection periods of more than 60 min, this effect was eliminated in the following way. The animal, after injection, was placed in a 400-ml beaker, the bottom of which was covered with screen wire. Food and water were freely available. About 45 min before termination of the experiment, the mouse was caught, induced to urinate into the above noted beaker, and then the penis was ligated. The preceding maneuver and ligation is difficult, and must be done with dexterity; with practice one can expect reasonable assurance of success.

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A single intravenous injection of inulin solution in the amount of 0.01 ml/g body weight with a rate of approximately 0.02 ml/sec was given, using a 0.25 ml serological syringe with a 27-gauge needle 0.5 in. long. The time interval for clearance begins at the moment injection is complete. For the average 20-g mouse, the quantity injected was 0.2 ml containing 1.0 μCi/254 μg. After injection, the animal was placed in a beaker as noted above and left undisturbed with food and water present until time of sacrifice or ligation. The animal, at the appropriate time, was sacrificed and heart blood was collected immediately in a vial after spreading about the edge of the vial 1 drop of heparin (1000 units/ml) from a 25-gauge needle. In the case of animals observed for clearance periods of 60 min or less, the intact bladder was carefully dissected free, removed, placed in a 50-ml volumetric flask, punctured, carefully rinsed with approximately 10 ml, and then made up to volume with water.

For the animals ligated during a part of the observation period, the dissected bladder was placed in a 100-ml volumetric flask and rinsed as previously described. The beaker containing the previously voided urine was rinsed four or five times. The washings were added to the contents of the 100-ml flask and made up to volume. This procedure is repeated for each mouse. For each experimental point, six mice are used, and the individual measurements are averaged.

A 1-ml aliquot of the urinary bladder washings either from the 50-ml dilution, or the 100-ml dilution was pipetted into a scintillation vial, to which 15 ml of scintillation fluid (B) was added and the vial tightly capped. After 5 min of agitation on a high-speed shaker, this material is ready for counting.

The blood sample was prepared as follows: 0.1 ml whole blood was pipetted into a scintillation vial to which 0.5 ml of 0.2 *N* NaOH was added, and the vial tightly sealed to prevent volatilization. After digesting approximately 48 hr at 80° and cooling to room temperature, the sample was bleached by adding 0.1 ml 30% H_2O_2. When bleaching was complete, 0.3 ml of H_2O was added. Then 15 ml of scintillation fluid (A) was added to the sample and the mixture agitated vigorously on a high-speed shaker for 5 min.

The background samples were prepared exactly as above with urine and blood obtained from noninjected mice. For blood standards, the 0.3 ml water was replaced with ^{14}C-inulin equivalent to the dose injected per 1 g observed body weight. For the urine standards, ^{14}C-inulin was added to the flask before bringing it up to a volume where 1 ml contained an amount equal to the dose injected per gram body weight.

When the entire experiment was completed, all samples, including backgrounds and standards, were counted for 1 min each. The settings of the spectrometer were 50–1000 discriminator and 6.5% gain. The dpm for the urine standard was 63,764 and for the blood 46,671. The total body doses were calculated separately for urine and blood and the relative dpm were used for our estimation.

Calculations. The data were calculated by determining ^{14}C-inulin excretion values derived from a curve showing cumulative inulin excretion with time. This curve is constructed to show percentage of total body dose in dpm. The blood estimation was made from inulin time-concentration curves of a similar nature.

Clearance periods begin at 10 min after inulin injection, for by this time the blood inulin concentration time curve permits valid midpoint blood estimation for any given time interval. The validity of the above assumption is readily seen when one examines Fig. 1. The clearance in milliliters/min is obtained by dividing the estimated urine excretion dpm per cent/min by midpoint blood inulin in dpm per cent/ml. To compare the data from the mice to that of man, the data for mouse surface area was calculated, using Meeh's formula (3): $SA = 0.114\ \sqrt[3]{W^2}$, where SA is the surface in square meters, and W is the observed weight of the animal in kilograms. The number 0.114 is the characteristic constant for mice as calculated by Rubner (3). The resulting figure was then extrapo-

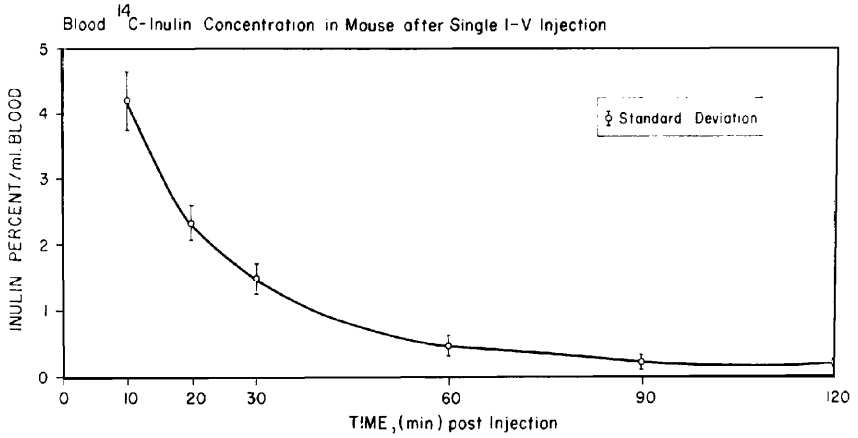

FIG. 1. Blood inulin concentration time curve established from six groups of six mice. The narrow limits of the standard deviation beyond 10 min permits valid midpoint blood estimation for any given time interval.

lated to a 1.73 m^2 surface area by multiplying the renal clearance in the mouse in milliliters/min. (UV/B) by 1.73/0.114 $W^{2/3}$. For the average mouse of 20 g, the surface area was calculated to be 0.0084 m^2 and the factor for extrapolation to the surface area of a standard man is 206.0

Results. Experimental data are given in Tables I and II and Fig. 1. To our delight, we have found this procedure for determining inulin clearance in a mouse to be highly reproducible. The average inulin clearance for a 20-g mouse was found to be 0.491 ml/min with a range of 0.435–0.607. The standard deviation was ±0.043. The mean renal clearance of inulin in the mice observed by us and extrapolated to a surface area of 1.73 m^2 to compare with a normal human subject is estimated to be 101 ml/min. Hawk stated that the inulin whole blood clearance in the average normal standard man is 100–125 ml/min (4). Thus, it is seen the mouse inulin clearance as extrapolated, is in very good agreement with inulin clearance values for man on a surface area basis. We have good reason, therefore, for assuming that in the mouse we have obtained a satisfactory estimate of glomerular filtration rate.

Discussion. The glomerular filtration rate remains quite constant under most conditions

TABLE I. Urinary ^{14}C-Inulin Excretion in Mice, Percentage of Injected Dose Over Various Time Intervals.

	0–10 min	0–20 min	0–30 min	0–60 min	0–90 min	0–120 min
	54.02	59.36	78.57	86.98	90.85	96.19
	39.97	85.77	74.09	88.93	94.34	101.06
	40.80	66.07	79.55	84.40	100.00	99.03
	46.40	55.46	72.17	86.99	90.53	95.16
	37.40	79.73	81.94	94.89	94.06	100.00
	62.47	52.10	75.86	87.69	95.24	—
Average	46.68	66.42	77.03	88.31	94.17	98.29
SD[a]	9.83	13.61	3.65	3.55	3.45	2.52
SE[a]	4.92	5.56	1.49	1.45	1.41	1.13

[a] SD = standard deviation of the mean; SE = standard error of the mean. The 50% excretion is at 11 min, determined by interpolation. Values represent individual determinations for six mice per experimental point.

TABLE II. ^{14}C-Inulin Clearance Calculation in Mice.

Collection period (min postinj.)	Interval inulin excretion		B Blood (% ml)	UV/B Clearance (ml/min)	UV/B · (1.73/0.114 $W^{2/3}$)
	% Period	UV (% min)			
10–20	19.8	1.98	3.263	0.607	125.0
20–30	10.0	1.00	1.907	0.524	107.9
30–40	6.0	0.60	1.223	0.491	101.2
40–50	4.1	0.41	0.830	0.494	101.8
50–60	2.6	0.26	0.583	0.446	91.9
Average				0.512	105.6
10–30	29.8	1.490	2.632	0.566	116.6
20–40	16.0	0.800	1.580	0.506	104.2
30–50	10.1	0.505	1.038	0.487	100.3
40–60	6.7	0.335	0.712	0.470	96.8
50–70	4.6	0.230	0.526	0.437	90.0
Average				0.493	101.6
10–40	35.8	1.193	2.191	0.545	112.3
20–50	20.1	0.670	1.344	0.499	102.8
30–60	12.7	0.423	0.894	0.473	97.4
40–70	8.7	0.290	0.634	0.457	94.1
60–90	5.3	0.177	0.407	0.435	89.6
Average				0.482	99.4
10–50	39.9	0.997	1.872	0.533	109.8
20–60	22.7	0.567	1.164	0.487	100.3
30–70	14.7	0.367	0.793	0.463	95.4
Average				0.494	101.8
10–60	42.5	0.850	1.629	0.522	107.5
20–70	24.7	0.494	1.033	0.478	98.5
40–90	12.0	0.240	0.537	0.447	92.1
Average				0.482	99.4
10–70	44.5	0.742	1.446	0.513	105.7
30–90	18.0	0.300	0.662	0.453	93.3
Average				0.483	99.5
General average				0.491	101.2
Standard deviation				±0.043	

and therefore can be used as an excretory standard with inulin to determine excretion of radiopharmaceuticals. With radiopharmaceuticals, it is important to know both primary point of distribution and rate of excretion. If the blood volume is part of the primary distribution pool, the clearance rate gives data of great importance for calculation of dose in rads. If primary distribution is to another known pool, then hematologic radiation can be calculated quite accurately.

Other investigators (5) have used ^{14}C-inulin clearance in dogs to simultaneously evaluate the excretion of new radiopharmaceuticals. The information gained by such studies in dogs is equally available with our technique, and in addition, the reproducibility of the renal clearance in mice, and the opportunity for the study of large numbers of animals may permit the intercomparison of several compounds evaluated in sequence as well as simultaneously.

This procedure will be used as part of a screening program for new radiopharmaceuti-

cal compounds. Not only will information be obtained as to the rate of excretion, but also at times as to the mode of excretion, *i.e.*, by filtration alone, as in inulin. As pointed out by Smith (6, 7), when the observed clearance has a ratio to the inulin clearance of greater than 1.0, it may be accepted that the substance under study is excreted by the tubules in addition to being filtered through the glomeruli. While ideally, the clearances should be done simultaneously, we believe that clearances determined in sequence will provide information of nearly equal value. Since the renal blood flow is more labile than the fraction filtered, presumptive evidence may be gained by this method of nephrotoxic properties which affect the renal blood flow. Since in drug evaluation it is very desirable to have a maximum of information on renal effects, we are planning on extending these studies to develop procedures for measurement of total renal blood flow by using hippuran, Diodrast, or other totally excreted compounds. Additional data so obtained will then enable us to determine with accuracy both mode and rate of excretion. This will substantially enhance capability to determine nephrotoxic effects as well as general systemic effects that may be reflected in an altered renal blood flow. Information can also be obtained in these ways on the stability of the radioactive label moiety of the compound under study since the excretion pattern will be altered when stability is low. This, we anticipate, will be of considerable value in determining the advisability of using compounds after some time in storage.

Conclusion. ^{14}C-inulin clearance was determined in mice and found to be remarkably reproducible from animal to animal.

^{14}C-inulin clearance observations are valid indicators of glomerular function when simultaneously administered with other radioactively labeled pharmaceuticals. The reproducibility of ^{14}C-inulin clearances in mice and the opportunity for testing relatively large numbers of animals offers promise that the clearance of radiopharmaceuticals may be evaluated in sequence, making this technique a potentially valuable bioassay for radiopharmaceuticals.

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