Synthesis, Characterization, and *in vivo* Disposition of Iodinatable Polyethylene Glycol Derivatives: Differences *in vivo* as a Function of Chain Length

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SUMMARY

A series of iodinatable water soluble polyethylene glycol (PEG) derivatives were prepared for use as model hydrophilic drugs. Polyethylene glycol diamine 6000 was coupled to methyl *p*-hydroxybenzimidate, and PEG 1900- and PEG 5000-monomethyl ethers were coupled to tyramine and histamine. The derivatives underwent facile iodination with the chloramine-T reaction and were stable under a wide range of conditions. The larger derivatives showed rapid renal clearance, but the 1900 MW compounds underwent significant clearance via the bile.

Keywords: Biliary excretion, Glomerular filtration rate marker, Iodination, Polyethylene Glycol

INTRODUCTION

To facilitate studies on the *in vivo* disposition of particulate drug delivery systems, we sought to prepare an easily measurable, hydrophilic model drug. Our requirements were identical with those of a glomerular filtration rate (GFR) marker, namely, "a) physiologically inert and non-toxic, b) not protein bound, completely filterable at the glomerulus, c) not reabsorbed or secreted, d) not subject to destruction, synthesis, or storage within the kidney, ..., f) have constant clearance over a wide range of plasma concentrations[1]."

Polyethylene glycol (PEG, MW = 400-6000) appeared to be a logical candidate for such a compound since it has been proposed as a GFR marker and can be specifically derivatized. As a GFR marker in dogs, its clearance matched that of creatinine over a 2-fold range of plasma concentrations[2a]. Moreover, PEG's of various sizes have been used to estimate the pore size in the glomeruli of dogs[2b]. Renal clearance of PEG-1000 in rats was found to be consistently greater

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than inulin clearance, and to vary with PEG plasma levels,[2c,d] however the markers still appear useful to follow renal excretion.

PEG can be derivatized specifically via the two terminal hydroxyls (one on the mono-methyl ethers). The structure is chemically stable and it appears to be biologically inert. Its properties have been exploited in a variety of ways: PEG-400 has been activated with phosgene and coupled with procaine to yield a longer acting aneasthetic[3]; PEG-6000 has been activated with cyanuric chloride and coupled with bovine serum albumin to reduce antigenicity[4]; PEG-10,000 has been activated with carbonyldiimidazole or dicyclohexylcarbodiimide and coupled to various amino acids as a soluble support for peptide synthesis[5]. In addition, the hydroxyl groups can be readily converted to amino functions for alternative coupling schemes[6].

We prepared phenolic and histamine containing PEG derivatives that could be iodinated with chloramine-T as the final step in the synthesis. The iodinated PEG compounds are easily purified by anion exchange or gel filtration, thus any desired isotope of iodine can be introduced for various applications. The synthesis, characterization, and disposition as a function of PEG molecular weight is described herein.

MATERIALS AND METHODS

Polyethylene glycols were obtained from Polysciences, Inc., Warrington, PA. Ion exchange resins and some chromatography supports were obtained from Bio-Rad, Richmond, CA. Thin layer chromatography plates were silica gel, hard layer plates from Analtech, Newark, DE. Sephadex chromatography supports were obtained from Sigma Chemicals, St. Louis, MO. Carbonyl-diimidazole was purchased from Pierce Chemical Company, Rockford, IL. Tritiated PEG MW 900 and MW 4000 were obtained from New England Nuclear, Boston, MA as a dry powder and were dissolved in phosphate buffered saline. All other chemicals and solvents were reagent grade or better. Ultraviolet spectra were recorded on a Cary-18. NMR spectra were recorded on a Varian FT-80 using D_2O or $CDCl_3$ containing 3-(trimethylsilyl)-1-propanesulfonic acid (Aldrich, Milwaukee, WI) or tetramethylsilane as internal standard. Three TLC solvent systems were used: A-chloroform/methanol/ammonium hydroxide/water 34/14/1/1; B-chloroform/methanol/acetic acid/water

34/14/1/1; and C- x% methanol in chloroform. TLC plates were routinely developed in iodine vapor, which is strongly adsorbed by PEG, and with one of the following sprays: $CoCl_2$, 1% in acetone; ninhydrin, 1% in acetone; or Folin reagent.

Polyethylene Glycol Diamine 6000 (2) -- 2 was prepared according to Mutter[7]. Assay of 2 with fluorescamine[8] showed quantitative conversion to the diamino compound. TLC system A, R_f = 0.9, detected with ninhydrin.

Polyethylene Glycol 6000 Bis (p-hydroxybenzamidine) (3) -- A mixture of 56.9 mg (9.5 mol) 2 and 4.3 mg (23 mol) methyl p-hydroxybenzimidate, hydrochloride[9], was suspended in 1 ml of chloroform. Addition of 20 l (140 mol) triethylamine gave a clear solution, which was stirred overnight at ambient temperature. The product was precipitated with 6 ml diethyl ether, and, after recrystallization from chloroform/diethyl ether, gave a white solid (53.4 mg, 91% yield). TLC system B, R_f = 0.95, and system C10, R_f = 0.1, detected with Folin reagent. UV: $_{max}$ 255 nm, = 10,700, NMR: 7.32(d x d), 3.69(s).

Polyethylene Glycol 6000 Bis(3,5-diiodo-4-hydroxybenzamidine) (4) -- Compound 3 (25 mg, 4.5 µmol) was dissolved in 100 µl borate buffer (100 mM, pH 8.0), and mixed with 32 µl (32 µmol) 1 M potassium iodide. Addition of 0.64 ml (32 µmol) chloramine-T(50 mM in borate buffer), turned the reaction briefly brown, then pale yellow. After 30 min at ambient temperature, the reaction was quenched by the addition of 0.5 ml(100 µmol) sodium bisulfite(200 mM). Gel filtration on Bio-Gel P-2(1.5 x 27 cm), eluting with water, yielded 4 in the void volume. Lyophilization gave 48.2 mg of a white powder, quantitative recovery. TLC system B, R_f = 0.95, and system C10, R_f = 0.1, detected with Folin reagent. UV: $_{max}$ 318 nm, = 11,4000, NMR: 8.18(s), 3.64(s).

Polyethylene Glycol 5000 Carbonylimidazole Methyl Ether (7a)[10] -- Dry PEG 5000 monomethyl ether (6a, 2.15 g, 0.43 mmol) was dissolved in 5 ml dichloromethane. The sodium salt of imidazole was prepared by the action of 35 mg(1.5 mmol) freshly cut sodium on 122 mg(1.8 mmol) imidazole in 10 ml dry THF.

After addition of 352 mg(2.2 mmol) carbonyldiimidazole and 0.5 ml(o.09 mmol) sodium imidazolide solution, the mixture was stirred overnight under nitrogen. The solvent was evaporated and the white solid was crystallized from chloroform/diethyl ether to give 2.36 g of a white powder.

Polyethylene Glycol 5000 2-(p-Hydroxyphenyl)ethylcarbamate Methyl Ether (8a) -- Tyramine (79 mg, 0.58 mmol), dissolved in 3 ml warm, absolute ethanol, was added to 1 g (0.19 mmol) 7a and 80 µl (0.57 mmol) triethylamine in 4 ml dichloromethane. After stirring overnight, the solvent was removed to give a pale yellow solid (quantitative yield). The crude product was purified in several batches by chromatography on BioGel P-6, eluting with water and lyophilizing the fractions. Final yield, 79% white powder. TLC system C50, R_f = 0.9, detected with Folin reagent. UV: λ_{max} 276 nm, ε = 1373, NMR: 7.1(d), 6.78(d), 3.64(s), 2.72(t), 1.30(t).

Polyethylene Glycol 5000 2-(3,5-Diiodo-4-hydroxyphenyl)ethylcarbamate Methyl Ether (11a) -- Compound 8a was iodinated as for 3, purified on P-2(1.0 x 28 cm) in borate buffer(100 mM), desalted on P-2, and lyophylized to give 195 mg white powder, quantitative recovery. TLC system C50, R_f = 0.9, detected with Folin reagent. UV: λ_{max} 291 nm, ε = 2066, NMR: 7.49(s), 7.17(s), 3.64(s), 1.23(t).

Polyethylene Glycol 1900 Carbonylimidazole Methyl Ether (7b) -- To 530 mg (0.28 mmol) dry PEG 1900 monoethyl ether (6b) in 2 ml dry CH_2Cl_2 was added 75 mg (0.46 mmol) carbonyldiimidazole and 10 mg (0.11 mmol) imidazole, sodium salt. After stirring overnight, 6 ml CH_2CL_2 was added and the mixture was extracted with 3.75 ml water, then dried with anydrous sodium sulfate. After filtration, solvent was removed (quantitative yield). Alternatively, the solvent was removed, and the resulting oil was recrystallized from chloroform/diethyl ether at -20°C. The white crystals were filtered through a chilled funnel, rinsed with a little diethyl ether, and used immediately.

Polythylene Glycol 1900 2-(p-Hydroxyphenyl)ethylcarbamate Methyl Ether (8b) -- To 193 mg (0.1 mmol) 7b in 2 ml dichloromethane was added 31 mg (0.23 mmol) tyramine in 3 ml absolute ethanol, plus 57 µl (0.4 mmol) triethylamine. After stirring overnight, the solvent was removed. The resulting oil was taken up in 30 ml water, and extracted with 3 x 75 ml chloroform. The combined chloroform layers were dried over anhydrous sodium sulfate and evaporated to give 173 mg clear oil. This was purified by gel filtration on Sephadex G-25 (1.5 x 49 cm), eluting with water. Fractions from 26 to 44 ml were pooled and lyophilized to give 137 mg (68%) of a clear oil, pure by TLC. TLC system C20, R_f = 0.75, detected with Folin reagent. UV: λ_{max} 276 nm, ε = 1390, NMR: 6.88 (d x d), 3.63(s), 3.37(s), 2.70(t), 1.30(t).

Polyethylene Glycol 1900 2-(3,5-Diiodo-4-hydroxyphenyl)ethylcarbamate Methyl Ether (11b) was prepared in a similar fashion to 11a.

Polyethylene Glycol 1900 2-(4-Imidazolyl)ethylcarbamate Methyl Ether) (9) -- To 600 mg (30 µmol) 7b in 1 ml dichloromethane was added 50 mg (0.45 mmol) histamine in 1.5 ml ethanol and 70 µl (0.5 mmol) triethylamine. After stirring overnight, the solvent was removed. The oil was taken up in 5 ml dichloromethane and extracted with 5 x 1 ml 0.5 M Na_2HPO_4 to remove histamine. An NMR spectrum taken after the third extraction was virtually identical with a spectrum taken after the fifth extraction, showing one histamine per PEG. The product was pure by TLC, 79% yield. TLC system A, R_f = 0.9, and system C50, R_f = 0.2. detected with Folin reagent. NMR: 7.57(s), 6.81(s), 3.63(s), 3.36(s), 2.79(t), 1.30(t).

^{125}I-Iodinations (to give 5, 11a,b, and 13) -- To 1.5 mg (0.3 - 0.7 µmol) substrate (0.3 - 0.7 µmol) substrate (3, 8a,b and 9 respectively) in 0.1 ml 100 mM borate buffer (pH 8.0) was added 3 µl (300 µCi, 15 pmol) $Na^{125}I$ [New England Nuclear, Boston, MA] and 10 µl (14 nmol) 50 mM Chloramine-T, then kept at room temperature for 15 min. Addition of 10 µl (390 nmol) 200 mM sodium bisulfite stopped the reaction. The reaction mixture was purified on a small (5 x 70 mm) AG1-X4-Cl- column, eluting with normal saline. The first 1-2 ml contained the

compound, while 99.95% of $^{125}I^-$ was retained on the column. Typical reactions incorporated 60-80% of ^{125}I. The products were judged pure by a second anion exchange column, a Bio-Gel P-2 column, and by TLC.

Stability Studies - The new compounds were tested for stability by incubating at 37°C in buffers(100 mM) at pH 2.0, 4.5, 7.4, or 11.0 for 18 days, or with mouse liver homogenate or lysosomes for 24 h.

Liver Homogenate and Lysosomes - Four mouse livers (6.4 g) were homogenized in 20 ml chilled(4°C) Tris/sucrose buffer (10 mM Tris/HCl, pH 7.5, 250 mM sucrose, 0.1 mM toluenesulfonyl fluoride as antipeptidase). The homogenate was assayed for protein by the modified Lowry method[11].

Half of the homogenate was centrifuged for 12 min at 1000 x g. The supernatant was centrifuged at 4°C for 30 min at 20,000 x g. The pellet was resuspended in 6 ml sodium acetate buffer (150 mM, pH = 4.6), assayed for protein, and frozen at -40°C. The lysosomes were warmed to 37°C and refrozen three times to lyse the membranes, releasing lysosomal enzymes.

A typical assay is described below: 6.5 µg(10^6 cpm) ^{125}I-5 was incubated with 200 µl liver homogenate or lysosomal enzymes(12 mg or 4 mg protein respectively) in a sealed centrifuge tube at 37°C. After 0,1,2,4,8, and 24 h, aliquots(10 µl) were spotted in triplicate on a TLC plate. The plates were developed in 1 N HCl/2.5% potassium iodide[12] to resolve radioactive $^{125}I^-$ (R_f = 0.9), free diiodobenzamidine (R_f = 0.7), and intact polymer (R_f = 0). Plates spotted with 11a or 11b were developed in chloroform/methanol/acetic acid/water: 34/14/1/1 to resolve intact marker (R_f = 0.9), $^{125}I^-$ (R_f = 0.6), or diiodotyrosine (R_f= 0). Autoradiography of the TLC plates showed very little breakdown, which was quantified by scraping and counting the bands.

pH Stability - A mixture of 1.0 mg(160 nmol) 4 and 20 µl(2 x 10^6 cpm) ^{125}I-5 in 130 µl 100 mM buffer was kept at 37°C for 25 h in a capped micro-centrifuge tube. The reactions were sampled at 0,1,2,4,8, and 24 h, spotted on TLC plates, and analyzed as above. Single values were taken for each time point. The

buffers used were: pH 2-HCl/KCL; pH 4.5-acetate; pH 7.4-phosphate; and pH 11.0-borate.

Organ Clearances - White ICR mice [Simonsen, Gilroy, CA], 25-30 grams, were fed and watered ad libitum, and kept on a 12 h per day light cycle. Each animal was injected via the tail vein with 0.1 ml per 10 g body weight(14% of normal blood volume, assuming 7.3% by weight). A typical dose contained 2.5 µg 4 and 200,000 cpm ^{125}I-5 per 0.1 ml. Three or four animals were injected per time point. Each animal was anesthetized with chloroform, and, after collecting 0.5 ml blood by cardiac puncture, the desired organs were dissected, rinsed with water, weighed and counted. After carefully removing the bladders, the carcasses were rinsed with water and counted.

Total Body Clearance - Three or four female, ICR mice were kept in a single metabolic cage [Model A4565, American Scientific Products, McGaw Park, IL] for 24 h. After 1,2,3,4,8, and 24 h, the urine and feces were collected. Water rinses of the cage were sampled, and counted (included with urine values). The entire feces were counted. Tritium containing feces were soaked overnight in 0.5 ml 1N potassium hydroxide, mixed with 0.5 ml t-butylhydroperoxide, and soaked overnight again. All tritium containing samples were mixed with 15 ml PCS [Amersham, Arlington Heights, IL] and counted. Radioactivity was determined using a Beckman LS-7800 scintillation counter or a Beckman Gamma-8000 gamma counter.

Bile Duct Cannulation - The femoral vein and bile duct of a female, Sprague Dawley rat [Simonsen, Gilroy, CA] (under light ether anethesia) were cannulated. Injections (0.5 ml saline containing the marker) were made via the femoral cannula, and bile was collected. The bladder was not catheterized, but urine was collected when possible. The rat, maintained for 22 h without anesthetic, appeared not to be under duress. Subsequent doses were given after 3.5-5 h, when < 0.1% of the last dose could be detected. The clearance of 13 was essentially the same whether given as the initial dose of after 18 h.

RESULTS AND DISCUSSION

The preparation of the derivatives, outlined in Figure 1, was straightforward. The larger PEG derivatives could be precipitated with diethylether from chloroform solutions, which facilitates intermediate purification. The PEG-1900 derivatives were very soluble in dichloromethane, while almost all impurities were more water soluble, leading to easy purification by extraction. All derivatives were purified by gel filtration and lyophilization prior to UV or NMR analyses.

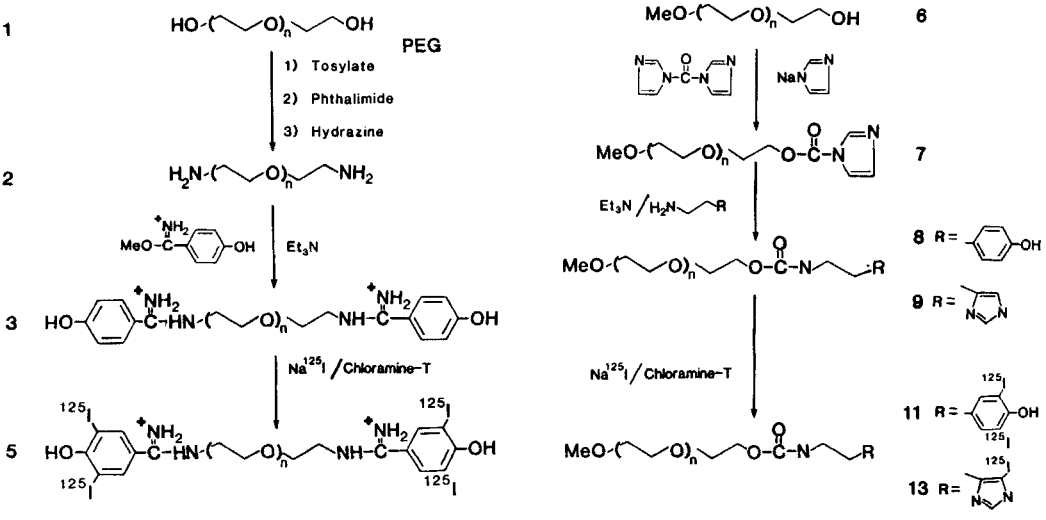

FIGURE 1: SYNTHETIC SCHEME AND STRUCTURE OF PEG DERIVATIVES

Two derviatives were tested for stability in liver homogenate (pH 7.5) or disrupted lysosomes(pH 4.6) at 37°C for 1 day. Very little breakdown of marker to either free I^- or free reporter group (e.g. diiodo-benzimidate or -tyramine), was observed (Table 1). Derivatives 4 and 9a were very stable, being essentially unchanged after incubation in 100 mM buffers at several pH's at 37°C for 18 days.

All of the derivatives were rapidly cleared from the body (Table 2). Table 2 lists the amount(%) of dose remaining in several tissues after various times. Each blood clearance curve could be described by a two term exponential decay, with half lives of 5-10 minutes (initial) and 200-300 minutes (terminal).

TABLE 1: STABILITY OF PEG MARKERS UNDER VARIOUS CONDITONS[a]

Compound	pH 2.0[b]	pH 4.5[b]	pH 7.4[b]	pH 11.0[b]	Liver[c]	Lysosomes[c]
5	98.7	99.6	101.5	97.7	97	92
11a	100	ND	96.8	93.8	100	99
13	ND	ND	97[d]	ND	ND	ND

[a] % of compound unchanged(2-3 determinations, ± < 4%)
[b] after 18 days, 37°C
[c] after 24 hours, 37°C
[d] after 5 days, 37°C
ND = not determined

The larger derivatives, 5 and 11a, were cleared almost exclusively by the kidneys (Tables 2 and 3). Although the residual levels of marker in individual organs dropped rapidly, the amount remaining in the whole animal was judged to be excessive for use as a hydrophilic model drug compound when compared to ^{14}C-inulin (1.4% total *in vivo* after 5 hours)[13].

Decreasing the chain length of PEG to MW = 1900 led to substantial biliary excretion (Tables 3 and 4). Although PEG-400 has been reported to be quantitatively recovered in canine urine[2a], we found significant biliary excretion(12%) of ^{3}H-PEG-900 in the rat. Biliary clearance of 11b and 13 was several fold higher which must be a result of the head group. However, the head group probably does not dominate the process since ^{125}I-histamine was found to be 95% excreted in urine, 75% after 2 hours, with little evidence for excretion via the bile. Moreover, diiodophenols resemble several thyroid hormones, which are not significantly excreted. The attachment of tyramine to polyhydroxyethyl-aspartamide at a mole percentage of 20% results in both a more rapid and greater accumulation of the polymer in the kidney than the non-modified polymer[14]. *In vitro* experiments demonstrate that the phenolic residues enhance the rate of pinocytotic capture of the polyhydroxyethylaspartamide[15]. In our studies, the attachment of tyramine and histamine modified the *in vivo* distribution of only the 1900 MW PEG, not the 5000 MW, and the 1900 MW derivatives accumulated in the

TABLE 2: ORGAN DISTRIBUTION OF PEG DERIVATIVES IN MICE[a]

Hours[b]	N[c]	Blood	Liver	Spleen	Lung	Kidney	Stomach	Intestine	Carcass
5, Polyethylene Glycol 6000 Bis(3,5-^{125}I-diiodo-4-hydroxybenzamidine)									
1	12	0.89±0.04	0.61±0.03	0.01±0.001	0.07±0.02	1.19±0.18	0.24±0.08	0.62±0.03	7.10±0.37
4	3	0.282±0.03	0.31±0.04	0.02±0.01	0.05±0.02	0.42±0.07	0.23±0.17	0.51±0.55	ND
24	3	bkg	bkg	bkg	bkg	bkg	bkg	bkg	bkg
11a, Polyethylene Glycol 5000 2-(3,5-^{125}I-Diiodo-4-hydroxyphenyl)ethylcarbamate Methyl Ester									
0.5	4	2.98±0.16	2.30±0.51	0.06±0.01	ND	1.58±0.19	0.70±0.10	1.40±0.04	15.3±15.4
1	7	1.39±0.06	1.31±0.17	0.05±0.01	0.38±0.18	0.89±0.09	0.64±0.03	1.16±0.2	17.9±2.3
4	4	0.48±0.10	0.53±0.05	0.01±0.00	ND	0.60±0.18	0.53±0.34	0.40±0.13	4.9±1.5
11b, Polyethylene Glycol 1900 2-(3,5-^{125}I-Diiodo-4-hydroxyphenyl)ethylcarbamate Methyl Ester									
0.5	8	0.59±0.09	5.12±1.7	0.02±0.00	0.09±0.03	0.28±0.17	1.01±0.57	30.6±7.3	14.0±2.9
1	12	0.99±0.10	2.04±0.95	0.02±0.00	0.12±0.02	0.64±0.37	0.61±0.07	27.5±12.3	16.5±9.3
4	8	0.04±0.01	0.21±0.10	0.01±0.02	0.02±0.01	0.03±0.01	0.89±0.97	1.39±1.2	19.8±9.7
24	4	0.02±0.01	0.06±0.02	bkg	bkg	0.02±0.01	0.09±0.1	0.88±0.12	0.65±0.18
13, Polyethylene Glycol 1900 2-(4-^{125}I-Imidazolyl)ethylcarbamate Methyl Ester									
0.25	2	1.4,1.7	4.45,4.62	0.03,0.05	0.15,0.19	0.41,5.7	0.93,1.20	32.0,39.5	8.2,18.6
4	2	1.5,1.6	0.71,3.1	0.02,0.05	0.09,0.10	0.21,0.21	3.3,3.7	0.85,1.5	35.2,38.8

[a] % of dose remaining per organ (mean ± standard deviation)
Blood values are % of dose remaining in total blood compartment
[b] Hours after administration of dose
[c] Number of animals per determination
[d] Duplicates
ND = not determined, bkg = background

TABLE 3: CLEARANCE OF PEG DERIVATIVES FROM MICE[a]

Compound	% in Feces	% in Urine	Time to Peak (hr)
^{3}H-PEG	0.87	73.3	1
5	1.63	101.3	3
11a	0.87	73.3	3
11b	57.3	21.6	8
13	33.7	64.1	2

[a] Mice were kept in metabolic cages, output (% of dose) determined as outlined in MATERIALS AND METHODS
Values are from a single representative experiment

bile, not the kidney. Thus it appears the effect of modification of polymers by small ligands on the *in vivo* behavior of the polymers in a complex function of the ligand, polymer, and ligand/polymer ratio.

PEG's of moleclular weight 4000-6000 were initially chosen because inulin, a standard GFR marker, has a molecular weight of 5000. However, the fraction of the dose remaining *in vivo* at 5 hours appeared to us to be unacceptable for a useful model drug compound. The lower molecular weight PEG-1900 derivatives were prepared to assure total filtration in small animals, such as the mouse[2b]. Surprisingly, the reporter group (tyramine, histamine) altered the properties of the polymer to the point where the derivatives were cleared mainly in the bile. Biliary excretion of the PEG-1900 derivatives is very rapid, comparable at least

TABLE 4: PERCENT OF DOSE EXCRETED INTO THE BILE[a]

Compound	% in bile	Time to peak (min)	Collection period (h)	% in urine	Peak (h)
11b	68.0	15	3:40	4.3	5
13	43.1	7	5	35.7	3
^{3}H-PEG-900	12.1	7	4	44.8	0:20
13	56.5	7	3	33.5	4:30
11b	78.6	7-15	5	--	--

[a] Values are from a single experiment with consecutive runs

in time course to imino-diacetic acids used for liver imaging[16], which may make the 1900 MW PEG derivatives useful markers for studying liver function and bile formation.

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