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Studies on the *In Vitro* and *In Vivo* Release of Hormonal Steroids Bound to a Polymer Base

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Abstract.- Poly (ethylene) glycol (PEG) based hydrogels crosslinked with 1,1,1, tris (hydroxy methyl) ethane, in phase separated hydrogels and 1,2,6, hexane triol in non-phase separated hydrogels were used to study the characteristics of release of hormonal steroids testosterone, progesterone and estradiol. A rapid *in vitro* release of steroids was observed with 1M PEG-6000 non-phase separated gel. A dramatic slowing down in the *in vitro* release characteristics was observed in the case of phase-separated gels and with increasing crosslinking. The time for 50% release ($t^{0.5}$) for the steroids was of the order of 9.5h, 10.5h and 12h for 1.5M, 2M and 3M PEG-6000 hydrogels, respectively. The 3M PEG-6000 phase separated gels retained the same characteristics of release *in vivo* since only 2.0% of ^{3}H-progesterone was released by 72h. The data demonstrates that PEG based hydrogels with appropriate crosslinking can be used effectively for sustained release of steroids from the site of implantation.

Key words: Hydrogel, polymer, steroid, release of hormonal steroids.

INTRODUCTION

Hydrogels are hydrophilic polymeric materials which have the ability to swell in water (30-90%). They retain a significant amount of fluid within their structure but remain water insoluble (Boretos *et al.*, 1975). In recent years a number of synthetic hydrogels have been employed for biomedical purposes. Biologically active compounds can be permanently or temporarily immobilized within the hydrogels and therefore these hydrogels can then be utilized for controlled release of drugs both *in vivo* and *in vitro* (Baker *et al.*, 1974; Sommerville *et al.*, 1976). The examples for such drugs include fertility control agents (Mishell *et al.*, 1970; Roseman and Higuchi, 1970; Woodland *et al.*, 1973), anticaner agents (Yolles *et al.*, 1971), weight gaining agents in cattle (Leafe *et al.*, 1974) and for treatment of glaucoma and ulcers (Tanquary *et al.*, 1972).

Hydrogels have been synthesized as polymers and copolymers of acrylamide (Davis, 1974), N-vinyl pyrrolidone (Hopfenberg, 1976), hydroxymethyl acrylate (Good, 1978) and acrylic or methacrylic acid. Ethylene vinyl acetate copolymer has been used for the delivery of low molecular weight drugs such as nitroglycerin and scopolamine (Edelman *et al.*, 1985). The implantation of protein hormones was a significant advancement in drug release technology because of the failure of previous attempts of compaction (Deanesly *et al.*, 1937; Pybus *et al.*, 1938), and encapsulation by silicone rubber (Folkman *et al.*, 1964; Tatum *et al.*, 1969).

In this study we report the *in vitro* and *in vivo* release of tritiated hormonal steroids: testosterone (T), progesterone (P) and 17-β-estradiol (E) from dried, phase and, non-phase separated hydrogels prepared from polyethylene glycol (PEG) the phase separated gels used in this study consisted of a polymer network system based on hexamethylene di-isocyanate, polyethylene glycol and 1,1,1, tris (hydroxy methyl) ethane as a crosslinking agent. In non phase separated PEG-hydrogels, the polymer network was based on methylene diphenyl di-isocyanate cross linked with 1,2, 6 hexane triol.

MATERIALS AND METHODS

Preparation and processing of hydrogels

Hydrogels were prepared essentially using the methods described by Mishell *et al.*, 1970; Roseman *et al.*, 1970. The gel matrix in each case consisted of polyethylene glycol (PEG-6000) which was crosslinked to hexa-methylene di-isocyanate. The crosslinking was achieved by 1,1,1, tris (hydroxy methyl) ethane. The extent of crosslinking was regulated by varying the concentration of crosslinking agent. In this way 1M, 1.5M, 2M and 3M PEG-6000 hydrogels were prepared. The nomenclature of hydrogels was based on the relative molar concentration of various structural elements in a given polymer.

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For all the four hydrogels (1M, 1.5M, 2M and 3M PEG-6000), three cylindrical pieces of equal length (1 cm) and volume were cut from each block of polymer and soaked separately for 24h in a large excess of distilled water. The pieces were vacuum dried at 40°C for 7-8h and used in the release experiments. In the case of 1M PEG-6000 non-phase separated hydrogel only cuboidal blocks (1 cm^2) were used for release studies.

The vacuum dried hydrogels were used for loading the hormones onto the gels. A specific amount of radioactive steroid (1,2,6,7, ^{3}H-testosterone 96 Ci/m mol; 1,2,6,7 ^{3}H-progesterone 88 Ci/m mol or 2,4,6,6,7 ^{3}H-estradiol 94 Ci/m mol, Amersham International, U.K.) was used in 100 μl volume for loading. Initially the steroid was dissolved in ethanol/toluene mixture (1:9 v/v). After evaporation, the ^{3}H-steroid was reconstituted in 10 ml solution of ethanol/chloroform (1:1 v/v). The blocks of polymer (cylindrical and cuboidal) were placed in this solution for 24h at 37°C. Before starting the loading, 100 μl aliquot of ^{3}H-steroid solution was drawn for calculating the total amount of radioactivity used for loading and similarly 100 μl aliquote was used for measuring the radioactivity incorporated into the gel after 24h. Following loading, the blocks were vacuum dried and used in the release experiments.

In vitro release

All release experiments were carried out in 0.01M sodium phosphate buffer pH 7.2. The dried block loaded with hormone was placed in 250 ml buffer in the case of phase separated hydrogels and 20 ml buffer in case of non-phase separated hydrogels. The release of steroids was recorded at various time intervals under constant temperature and continuous shaking (100 rpm). At each time interval, in all the cases, 500 μl aliquote were drawn and the radioactivity was counted in Beckman Liquid Scintillation Counter LS-180.

In vivo release

For these experiments, the loaded polymer blocks were implanted under the skin on the dorsal side of male and female Sprauge Dawley rats (3 month old). The animals were sacrificed at the desired time interval, blood collected and radioactivity counted in 100 μl of plasma.

RESULTS

In vitro studies

The release of testosterone (^{3}H-T), progesterone (^{3}H-P) and estradiol (^{3}H-E) was studied in both non-phase separated and phase separated 1M-PEG 6000 hydrogels. In case of non-phase separated hydrogels, the release of all the three steroids followed an identical pattern. The percentage of release of each steroid is tabulated in Table I. By 120 min, 63% of ^{3}H-T, 64% of ^{3}H-P and 67% of ^{3}H-E were released. Fifty percent release ($t^{0.5}$) was obtained in 72 min, 85 min and 70 min. for ^{3}H-T, ^{3}H-P and ^{3}H-E respectively. However, in the case of phase separated hydrogel 1M PEG-6000, the release of steroids was much slower compared to the non-phase separated gel (Table II). The release of activity in non-phase separated hydrogels was complete (70%) within 2h, whereas only 42% of the activity was released by 5h in phase separated gels, and 85% total activity was released only by 30h. The rate of release and release ratios calculated for all the steroids showed a slow and gradual release of activity.

Table I.- ***In vitro*** **release of ^{3}HT, ^{3}HP, ^{3}HE from non-phase separated hydrogel 1M PEG-6000.**

Time (min)	% release[1]			release rate %/min[2]		
	^{3}HT	^{3}HP	^{3}HE	^{3}HT	^{3}HP	^{3}HE
20	25.1	19.8	20.4	1.25	0.99	1.02
40	32.3	29.4	32.6	0.80	0.73	0.81
60	43.6	38.0	43.4	0.72	0.63	0.75
80	52.5	46.2	55.3	0.65	0.57	0.69
100	61.5	54.6	57.4	0.61	0.54	0.51
120	63.0	64.0	67.2	0.52	0.63	0.56

[1]% release: represents the % of the ^{3}H-steroid released at the appropriate time.
[2]release rate: rate/min was obtained by dividing the % release by time.

When 1.5M PEG-6000 hydrogel (phase separated) was used in the release experiments, the steroids released slower (Table III) than in case of 1M PEG-6000 hydrogel. This is evident from the $t^{0.5}$ value of ^{3}H-T which is 6h in case of 1M PEG-6000 and 9.5h for 1.5M PEG-6000. The extent of total release was 80% by 30h.

In case of 2M PEG-6000 hydrogels the extent of total release was slower (75% by 27h, Table IV). The $t^{0.5}$ value for ^{3}H-T in this hydrogel was 10.5h, compared to 9.5h for 1.5M PEG-6000.

The extent of release of labelled steroid was even slower in case of 3M PEG-6000 compared to the other hydrogels tested. The $t^{0.5}$ value was found to be 12h for ^{3}H-T (Table V). The extent of total release was 71% by 27h. The release rate and release ratio calculated for all the hydrogels showed a slow and gradual release of activity.

Table II.- *In vitro* **release of ^{3}HT, ^{3}HP, ^{3}HE from phase separated hydrogel 1M PEG-6000.**

Time	% release[1]			release rate (% h)[2]			release ratio (Qt/Q∞)[3]		
(h)	^{3}HT	^{3}HP	^{3}HE	^{3}HT	^{3}HP	^{3}HE	^{3}HT	^{3}HP	^{3}HE
1	16.0	15.2	15.5	16.0	15.2	15.5	0.19	0.18	0.18
2	26.0	24.4	26.2	13.0	12.2	13.1	0.31	0.29	0.30
3	30.0	28.8	33.6	10.0	9.6	11.2	0.35	0.34	0.39
4	37.6	33.0	37.6	9.4	8.5	9.4	0.44	0.41	0.41
5	42.5	38.0	40.5	8.5	7.6	8.1	0.50	0.46	0.47
6	48.0	43.2	45.0	8.0	7.2	7.5	0.57	0.52	0.53
24	76.8	74.4	74.4	3.2	3.1	3.1	0.91	0.90	0.87
27	78.3	75.6	78.3	2.9	2.8	2.9	0.93	0.92	0.91
30	83.7	82.1	85.5	2.7	2.6	2.7	1.0	1.0	1.0

[1]% release: represents the % of the ^{3}H-steroid released at the appropriate time interval.

[2]release rate: rate/h was obtained by dividing the % release by time.

[3]Qt/Q∞: ratio of % release at time Qt, divided by % release at final time (Q∞).

Table III.- *In vitro* **release of ^{3}HT, ^{3}HP, ^{3}HE from phase separated hydrogel 1.5M PEG-6000.**

Time	% release[1]			release rate (% h)[2]			release ratio (Qt/Q∞)[3]		
(h)	^{3}HT	^{3}HP	^{3}HE	^{3}HT	^{3}HP	^{3}HE	^{3}HT	^{3}HP	^{3}HE
1	16.3	12.9	15.1	16.3	12.9	15.1	0.21	0.16	0.18
2	30.8	20.2	21.1	15.4	10.1	11.0	0.39	0.26	0.26
3	34.8	27.1	25.2	11.6	9.3	9.7	0.44	0.36	0.36
4	37.6	34.4	36.0	9.4	8.6	9.0	0.48	0.44	0.45
5	41.0	37.5	42.0	8.2	7.5	8.5	0.52	0.48	0.52
6	43.8	42.6	47.4	7.3	7.1	7.9	0.56	0.54	0.58
24	72.0	69.6	74.4	3.0	2.9	3.1	0.92	0.89	0.92
27	75.6	72.9	75.6	2.8	2.7	2.8	0.97	0.94	0.94
30	77.5	77.5	80.6	2.5	2.5	2.6	1.0	1.0	1.0

[1]% release: represents the % of the ^{3}H-steroid released at the appropriate time interval.

[2]release rate: rate/h was obtained by dividing the % release by time.

[3]Qt/Q∞: ratio of % release at time Qt, divided by % release at final time (Q∞).

In vivo studies

The release of ^{3}H-P bound to non-phase separated hydrogels was monitored from ^{3}H-P and ^{3}H-P and ^{3}H-T loaded subcutaneously implanted blocks in mature rats. The release of steroid (Table VI) followed a pattern of sustained release for a period of 5h, but was considerably slower compared to *in vitro* release. Only 3% of total steroid was release in the same time compared to 64% *in vitro* from the same gel.

When ^{3}H-T loaded 3M PEG-6000 hydrogel (phase separated) were implanted subcutaneously in mature rats, only 2.06% of the total activity was released after a period of 72h compared to 70% from the same gel *in vitro* (Table VII).

There was no indication that *in vivo* implants caused any irritation in the area of implantation.

DISCUSSION

Although several previous studies have shown that hydrogels can be used effectively for slow release of drugs (Davis, 1974; Ebert *et al.*, 1980; Graham *et al.*, 1980; McNeill *et al.*, 1984), however, the release characteristics vary with the nature of the drug and the

Table IV.- ***In vitro*** **release of ^{3}HT, ^{3}HP, ^{3}HE from phase separated hydrogel 2M PEG-6000.**

Time	% release[1]			release rate (% h)[2]			release ratio (Qt/Q∞)[3]		
(h)	^{3}HT	^{3}HP	^{3}HE	^{3}HT	^{3}HP	^{3}HE	^{3}HT	^{3}HP	^{3}HE
1	13.2	14.0	13.6	13.2	14.0	13.6	0.16	0.18	0.18
2	20.7	12.2	21.2	10.3	10.6	11.1	0.25	0.28	0.24
3	28.8	27.0	30.3	9.6	9.0	10.1	0.35	0.36	0.40
4	33.3	32.3	32.4	8.3	8.0	8.1	0.41	0.43	0.43
5	38.0	36.0	37.5	7.6	7.2	7.5	0.46	0.48	0.50
6	42.0	38.4	41.4	7.0	6.4	6.9	0.51	0.51	0.55
24	72.0	67.2	65.0	3.0	2.8	2.7	0.88	0.90	0.87
27	75.6	70.2	71.0	2.8	2.6	2.6	0.93	0.94	0.95
30	81.0	74.4	74.0	2.6	2.4	2.3	1.0	1.0	1.0

[1]% release: represents the % of the ^{3}H-steroid released at the appropriate time interval.

[2]release rate: rate/h was obtained by dividing the % release by time.

[3]Qt/Q∞: ratio of % release at time Qt, divided by % release at final time (Q∞).

Table V.- ***In vitro*** **release of ^{3}HT, ^{3}HP, ^{3}HE from phase separated hydrogel 3M PEG-6000.**

Time	% release[1]			release rate (% h)[2]			release ratio (Qt/Q∞)[3]		
(h)	^{3}HT	^{3}HP	^{3}HE	^{3}HT	^{3}HP	^{3}HE	^{3}HT	^{3}HP	^{3}HE
1	8.3	11.7	9.3	8.3	11.7	11.3	0.11	0.16	0.13
2	15.2	20.6	16.1	7.6	10.3	8.0	0.20	0.29	0.22
3	22.2	28.8	21.6	7.4	9.6	7.2	0.30	0.40	0.30
4	28.4	36.8	26.0	7.1	9.2	6.5	0.38	0.51	0.36
5	32.5	41.0	29.4	6.5	8.2	5.8	0.44	0.57	0.41
6	38.0	45.6	31.1	6.3	7.6	5.3	0.51	0.74	0.45
24	66.5	62.4	61.5	2.7	2.6	2.5	0.90	0.88	0.86
27	71.8	64.8	64.6	2.6	2.4	2.3	0.97	0.91	0.90
30	73.4	70.9	71.1	2.3	2.2	2.2	1.0	1.0	1.0

[1]% release: represents the % of the ^{3}H-steroid released at the appropriate time interval.

[2]release rate: rate/h was obtained by dividing the % release by time.

[3]Qt/Q∞: ratio of % release at time Qt, divided by % release at final time (Q∞).

polymer matrix used in the gel. We have studied *in vitro* and *in vivo* release profiles of steroids: testosterone, progesterone and estradiol from both phase separated and non-phase separated PEG-hydrogels. Our data clearly demonstrate that non-phase separated gels have rather poor characteristics for the release of the three steroids, since bulk of the steroid (70%) was released in a single chunk during the first hour. There was no difference in the release pattern of any of the steroids studies. These data have been obtained on dried gels; in case of fully swollen gels, the release of drugs have been shown to be even faster (McNeill *et al.*, 1984).

A dramatic slowing down in the release of steroids was observed when phase separated hydrogels were used. It is generally agreed that water in these gels is in bound and free forms (Jhon *et al.*, 1973; Kim *et al.*, 1980). And that the bound water is associated with the ether group of the gel in crystalline state. The delay in the release of the drug is determined by the extent of bound water to the gel. It appears that phase separation in our experiments increased the concentration of bound water thus causing a delay in the release of steroids. Our data on the release of steroids *in vitro* also demonstrate that the delay in the release of steroids is

Table VI.- *In vivo* **release of ^{3}H-progesterone from non-phase separated hydrogel 1M PEG-6000.**

Time (h)	% release[1]	Release rate (%/h)[2]
0.5	1.60	3.20
1.0	1.70	1.70
1.5	1.86	1.24
2.0	2.07	1.03
3.0	2.18	0.72
4.0	2.54	0.63
5.0	2.81	0.56

[1]% release: represents the % of the ^{3}H-steroid released at the appropriate time interval.

[2]release rate: rate/h was obtained by dividing the % release by time.

Table VII.- *In vivo* **release of ^{3}H-testosterone from phase separated hydrogel 3M PEG-6000.**

Time (h)	% release[1]	Release rate (%/h)[2]
2	0.17	0.087
4	0.28	0.071
8	0.52	0.066
24	1.44	0.060
48	2.05	0.042
72	2.06	0.028

[1]% release: represents the % of the ^{3}H-steroid released at the appropriate time interval.

[2]release rate: rate/h was obtained by dividing the % release by time.

directly related to the the degree of crosslinking of the hydrogels. The delayed release therefore may be attributed to two factors: firstly, the content of bound form of water which increases with increasing crosslinking, e.g. more water molecules become bound to the ether group in 3M PEG-6000 than to 1M PEG-6000, secondly, with increasing corsslinking, the pore size of the gel decreased. Both these factors have obvious implication in the release profile of steroids. It may be noted that whereas 85% of the steroid is released after 30h from the 1M-PEG gel, the release from 1.5M, 2M and 3M PEG-6000 gels specifically decreased in the same order during the same time interval. It has been suggested that PEG-hydrogel have three domains: A, B and C. The 'A' domain relates to PEG, 'B' domain is contributed by the crosslinking and the 'C' domain comprised of free water. In the 3M PEG-6000 gel, the 'C; domain is minimal. For this reason, it can be argued that 3M PEG-6000 with high corsslinking, small pore size, more bound water content, combines the chracteristics which result in comparatively delayed release of the steroid in this series of gels.

In summary our data indicate that PEG based hydrogels, crosslinked with 1,1,1 Tris (hydroxy methyl) ethane can be used profitably for the slow *in vitro* and *in vivo* release of molecules like steroids.

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