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In vitro retinoid binding and release from a collagen sponge material in a simulated intravaginal environment

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Four *in vitro* preparations were constructed to simulate the intravaginal release of two retinoids, all-*trans*-retinoic acid (*t*-RA) and 13-*cis*-retinoic acid (*c*-RA), from a 0.7% collagen sponge diaphragm insert. Four *t*-RA concentrations, 0.019, 0.05, 0.1, and 0.15% in methanol were added to the sponge. The release into an artificial vaginal fluid was monitored serially over 72 h by serial analysis for *t*-RA and *c*-RA using high-pressure liquid chromatography. In each preparation, retinoid release was immediate and noncontinuous. At 37°C, the retinoids were stable for at least 48 h. *Trans*-retinoic acid was the predominant retinoid recovered. Only trace amounts of the *cis*-isomer were released. Peak *t*-RA levels were 20 μM after 0.01%, 60–80 μM after 0.05%, 100–200 μM after 0.1%, and 320 μM after 0.15%. When the vaginal fluid bath was changed after 5 h, no further significant retinoid release occurred. There was significant loss of up to 70% of the applied *t*-RA into the collagen sponge. The retinoid binding was concentration dependent (higher binding with higher concentrations) and was maximal only after 24 h of co-incubation. The discontinuous release of *t*-RA and the high degree of binding to collagen would seem to preclude use of the diaphragm insert as a vaginal drug delivery system, at least for retinoids.

INTRODUCTION

Vitamin A and, its natural and synthetic derivatives (retinoids) have demonstrated the ability to block the phenotypic expression of carcinogen-induced malignant transformation *in vitro*.[1-3] The effect is probably related to their natural role in controlling the differentiation of many epithelial tissues.[4,5] These potent biological effects as well as characteristic retinoid toxicities are amenable to structure activity modifications.[6] One such biologically active synthetic retinoid with a low toxicity index is 13-*cis*-retinoid acid, *c*-RA,[7,8] an isomer of the naturally occurring all-*trans* retinoic acid.

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Sporn et al.[2] has observed limited utility of retinoids when they were used as a potential cancer chemopreventative agent because of both toxicity and poor tissue distribution. It was hypothesized that direct mechanical delivery of an active retinoid to specific sites at risk of transformation could obviate the systemic toxicity.

The purpose of conducting the *in vitro* experiments described herein was to simulate as closely as possible a typical intravaginal environment to observe the binding and release characteristics of solutions of all-*trans*-retionoic acid, *t*-RA, from the collagen sponge diaphragm insert. This insert is a reconstituted bovine collagen wafer which has been used successfully to deliver drugs intravaginally to experimental animals and clinically as an experimental sperm-retentive diaphragm contraceptive.[8,9]

MATERIALS AND METHODS

Retinoids

Retinoids used in this study included 0.05% (by weight) all-*trans*-retinoic acid Tretinoin Liquid,* which also contains polyethylene glycol 400, butylated hydroxytolune, and alcohol 55%. This preparation was used at 0.05% (no dilution) and 0.01% (1:5 dilution in methanol). Other synthetic retinoids[†] were used. These included 100 mg of all-*trans*-retinoic acid (*t*-RA, MW = 300.4), 13-*cis*-retinoic acid (*c*-RA), and all-*trans*-retinol (MW = 286.5). Each was diluted in methanol to 2 mg/mL (6.65 μM *t*-RA and *c*-RA, and 6.98 μM retinol). The methanol used in the dilutions was 99.9% pure, chromatography grade.[‡] Stock solutions were used in assay development and generation of a standard curve for *t*-RA and *c*-RA and kept in sealed amber vials stored at 10°C. Initially, the Sigma *t*-RA was also diluted to 0.1 and 0.15% in methanol for *in vitro* addition to the collagen sponge.

Collagen sponges

Collagen sponges are made from pure collagen isolated from bovine skin swollen at pH 3.0 and reconstituted into the physical form of a sponge layer by the Chvapil procedure.[9] Glutaraldehyde is used as a crosslinking agent to provide resilience and stability of the sponge matrix in the acidic environment of the vagina. The average pore size was 400 A (range 80–1400 A). The sponges were provided in the form of thin round wafers approximately 3–4 mm thick and 7 cm in diameter. The mean dry sponge weight (n = 5) was 0.738 (SD = 0.042). It should be noted that this shape is substantially different from the larger cylindrical 2.5 × (6–7) cm contraceptive collagen sponge extensively researched by M. Chvapil.[10,11]

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† Sigma Chemical Co., St. Louis, MO.
‡ Burdick Jackson Laboratories, Muskegon, MI.

Simulated vaginal microenvironment

Four *in vitro* preparations were constructed, each in duplicate, to simulate the intravaginal release of *t*-RA and 13 *c*-RA from the collagen sponge. Preliminary testing demonstrated that 2.7 mL of a retinoid-containing solution in methanol completely wet a quarter-section of the sponge wafer. This standard volume was then used to wet quarter sponge sections with 0.01, 0.05, 0.1, or 0.15% solutions of *t*-RA. The wetted sponges, each in a 150 mL glass-stoppered beaker, were then brought to 33 mL final volume with an artificial vaginal fluid (AVF). The composition of this aqueous fluid (described in Table I) was designed to duplicate the electrolyte, nitrogenous, and pH conditions of the normal human vagina in an unstimulated state.[12-14] The retinoid-soaked sponges in sealed vials were immediately placed in a light protected shaker-water bath and kept at 37°C, 20 oscillations per minute for 72 h. Samples of the AVF were serially removed over time for retinoid analysis by high-pressure liquid chromatography (HPLC).

Samples for HPLC analysis were immediately diluted 1:1 with a solution containing 0.002% (69.8 μM) all-*trans*-retinol included as an internal standard. Duplicate samples were removed from the four preparations at the following times: zero; 15 min; 1, 2, 3, 4, and 5 h. Immediately after removing the 5-h samples, the AVF was drained and new solution was added. Subsequently, samples were withdrawn at 6, 12, 18, 24, 36, 48, 60, and 72 h. At 72 h, the remaining AVF was discarded and the moist quarter-sponge was removed and vigorously rinsed with several volumes of methanol up to 20 mL final volume. A sample was taken of this methanol rinse and the sponge discarded.

For HPLC analysis, 10-μL samples containing the internal standard 1:1 were injected onto a Varian Model 5020 high-pressure liquid chromatograph under the following conditions: isocratic run; mobile phase—85% methanol/15%

TABLE I
Composition of Artificial Vaginal Fluid (AVF)

Substance	Source	Concentration/L
Electrolytes		
KCl	Cutter multidose vial	23.5 mEq
NaCl	Abbott	39.0 mEq
Sodium Acetate	Abbott	22.0 mEq
Nitrogenous substances		
Albumin	Armour multidose vial	9.0 mg
Amino Acids	Travenol/8.5% amino acid injection	11.0 mg
Urea	MCB reagent grade	490 mg
pH		
Acetic Acid	Common vinegar (5%)	qs to 4.1
Fluid		
Distilled water	Baker HPLC water	qs 1 L

ammonium acetate 1% in water,* and flow rate, 1.2 mL/min. The column was a reverse phase C_{18}(10 μm particle size) column (½ in o.d., 4 mm i.d., 30 cm length).[†] It was kept at ambient room temperature. Absorbance was measured at 254 nm. Samples were stored at −10°C in an amber wrap for up to 1 week prior to analysis. Retinoid concentration was calculated by comparison of peak–height ratios (c-RA, t-RA to retinol) using c-RA-retinol and t-RA-retinol standard curves which were linear over the range 2.33–166 μM.

Retinoid binding to laboratory glassware and to the collagen sponge material was separately tested by placing one-quarter, one-half, and whole collagen sponge wafers into separate 0.005% t-RA–methanol solutions in amber glass vials. Samples were serially removed at time zero, 30 and 60 min, and 26 h for c-RA and t-RA analysis by HPLC.

RESULTS

A representative chromatograph (Fig. 1) demonstrates the separation of the retinoid isomers. Peaks were identified by co-elution with freshly prepared reference standards.

Figures 2 through 5 display the results of the HPLC analysis for t-RA and c-RA released from the quarter sections. The values represent the average of the duplicate tests run at each concentration. Analysis of log-retinoid
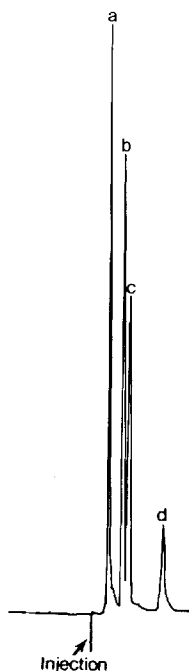

Figure 1. Chromatogram showing separation of (a) sodium phenobarbital, (b) 13-c-RA, (c) t-RA, and (d) retinol.
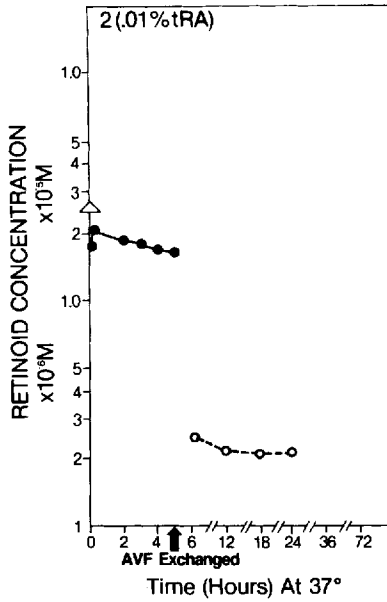
Figure 2. Time course of retinoic release froma a collagen sponge soaked in 0.01% *t*-RA. (●) All-*trans*-retinoic acid; (○) 13-*cis*-retinoic acid; (△) concentration if no binding occurred.

concentrations versus time revealed two consistent trends: (1) retinoid concentrations remained relatively low and constant over time; and (2) exchanging the AVF at 5 h (arrow on figures) removed most of the available *t*-RA. In each
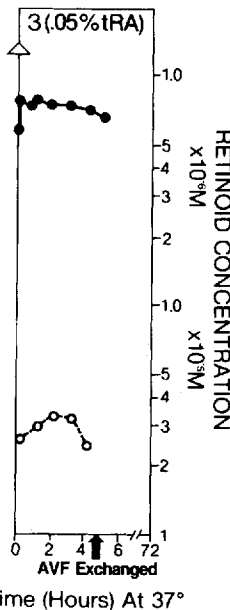

Figure 3. Time coure of retinoid release from a collagen sponge soaked in 0.05% *t*-RA.
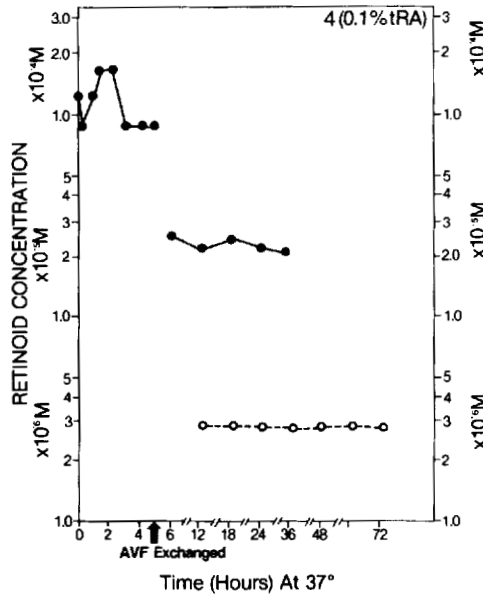
Figure 4. Time course of retinoid release from a collagen sponge soaked in 0.1% t-RA.

case, the total retinoids recovered from the AVF only accounted for 30–60% of the t-RA originally added to the sponge sections. Table II shows the results of attempts to extract sponge-bound retinoids from the experimental preparations at the end of 72 h. Retinoid binding increased proportionately with
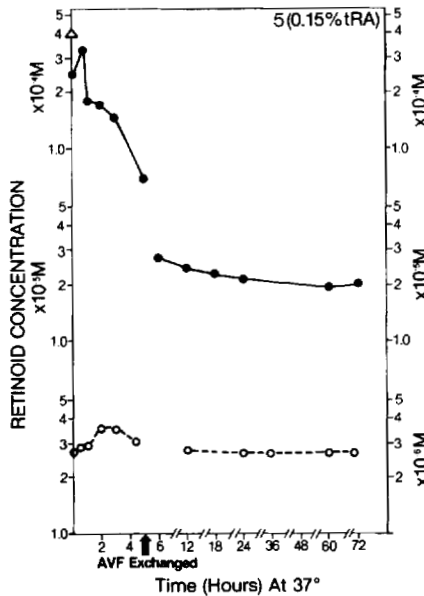

Figure 5. Time course of retinoid release from a collagen sponge soaked in 0.15% t-RA.

TABLE II

Recovery of *c*-RA and *t*-RA from the Collagen Sponge Immersed 72 h in AVF

Percent	*t*-RA vol. (ml)	*t*-RA added total (mg)	Quarter-collagen sponge weight (g)	Methanol eluted retinoic acid (mg)			
				c-RA	*t*-RA	Total	Total %
0.01	2.70	0.27	0.183	0.04	0.06	0.10	37.0
0.05 A	2.70	1.35	0.200	0.01	0.20	0.21	16.0
0.05 B	2.70	1.35	0.200	trace	0.14	0.14	10.3
0.1 A	2.70	2.70	0.186	0.08	0.29	0.37	13.7
0.1 B	2.70	2.70	0.186	trace	0.29	0.29	10.7
0.15 A	2.70	4.05	0.170	0.09	0.45	0.54	13.3
0.15 B	2.70	4.05	0.182	0.08	0.52	0.60	14.8

the t-RA concentration in contact with the quarter-sponge sections (Table II). The majority of recovered retinoids in these preparations were t-RA with very low concentrations of the 13-cis isomer found before and after the solution change at 5 h. Table II also displays the results of extracting the sponges for retinoids after 72 h. The 0.01% soaked sponge produced the largest proportional amount of methanol-recoverable retinoid (37% of the original concentration). In the other preparations, only small amounts of t-RA were removed by the extraction procedure (mean, 11.8%). The milligrams of retinoids recovered per gram of sponge weight increased in rough proportion (correlation coefficient of 0.81, p <0.05) with the t-RA concentration (Fig. 3).

The 13-c-RA isomer was not produced in a concentration dependent fashion. Concentrations ranged from 2–3 μM. The t-RA levels available from the sponge appeared to follow concentration-dependent changes in availability. Thus, the 0.01% preparation produced a mean level of approximately 16–20 μM, the 0.05% preparation reducing t-RA levels of 60–80 μM, roughly five times this amount. Following the solution change at 5 h, neither preparation yielded measurable t-RA levels. In contrast, the two higher concentration preparations (0.1 and 0.15%) produced significant postexchange t-RA levels; each on the order of 20–30 μM t-RA. Only the 0.15% preparation produced a significant t-RA decay pattern in which the concentration dropped from a peak of 320 μM to 68 μM over 4 h. In no preparation did the t-RA or c-RA concentrations increase over time.

The stability of t-RA in AVF at 37°C in glass vials was also investigated. Although there was very slight photoisomerization of t-RA to c-RA (approximately 5%), there was no significant loss of t-RA over a 48-h period.

Since it was known at the outset that retinoids have the potential for significant binding to plastic infusion system materials,[15] the sorption of t-RA to the collagen sponge was separately evaluated. Table III shows the results
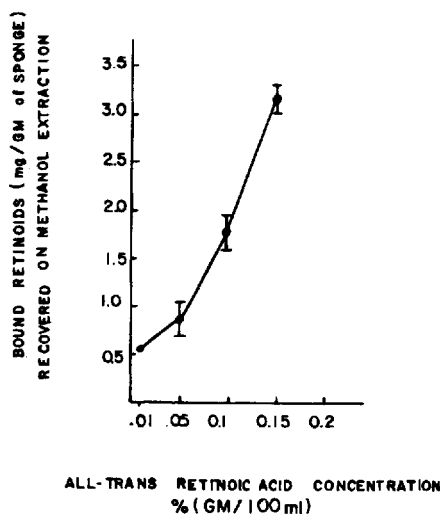

Figure 6. Methanol extractable retinoids bound per gram sponge weight (y axis) vs. t-RA concentration (x axis).

TABLE III
Retinoid Recovery from a Solution of 5 mg *t*-RA in 100 mL Methanol after Addition of Collagen Sponge

Time	Recoverable retinoids with quarter-sponge section (0.186 g)			Recoverable retinoids with half-sponge section (0.34 g)			Recoverable retinoids with whole sponge (0.73 g)		
	t-RA	*c*-RA	Percent recovered	*t*-RA	*c*-RA	Percent recovered	*t*-RA	*c*-RA	Percent recovered
Baseline (presponge addition)	4.42	0.58	100	4.75	0.45	100	4.50	0.50	100
Immediately upon sponge addition	4.02	0.48	90	4.28	0.52	96	4.46	0.54	100
30 Min	4.32	0.48	96	4.32	0.48	96	2.38	0.52	58
60 Min	2.64	0.56	64	3.50	0.50	80	1.92	0.48	48
26 H	1.70	0.50	44	1.78	0.62	48	0.60	0.74	27

of placing either one-quarter, one-half, or a whole collagen sponge into an identical solution of 0.005% *t*-RA in methanol and AVF (50:50). There was a time-dependent, but not surface area-dependent, reduction in retinoid concentration. After 60 min incubation at 20°C, 36, 20, and 52% of the total retinoids had been removed by the quarter, half, and whole collagen sponge, respectively. After 26 h incubation, the three sponge sections had adsorbed 56, 52, and 72.2% of retinoid, respectively. Thus, there was no significant difference in total retinoid sorption between the quarter- and half-sponge. In addition, and as earlier studies had predicted,[15] the binding of retinoids was not complete until after 24 h exposure to the absorbing surface.

DISCUSSION

This study shows the limitations of a collagen sponge biomaterial as an intravaginal drug delivery system. The results describe substantial binding of retinoid to the collagen. The bound retinoid was only partially recoverable by methanol rinsing. Total binding increased with time. The apparent release of retinoid from the collagen was rapid and there was no slow leaching of retinoids from the sponge into the aqueous medium. However, the retinoids appeared to be stable at the physiologic temperature and chemical environment of the simulated vaginal milieu as long as light exposure was minimized.

Preliminary *in vivo* studies with other drugs impregnated on this sponge diaphragm insert suggested direct communication of the sponge matrix with rabbit cervical mucus.[10] Another observation was that vaginal washout of some drugs, such as antibodies, zone, and mycostatic substances, might continue over 5–7 days.[16] In the current study, most of the available *t*-RA was immediately released into the AVF. It is possible that this was actually the result of two simultaneous processes: time-dependent penetration of drug into the collagen matrix competing with a concomitant slow release of drug solution out of the sponge and into the AVF. It is also apparent that significant *in vivo* turnover of vaginal fluid (simulated in this study by the solution change at 5 h) would result in a rapid lowering of any ambient retinoid concentrations.

Whether this *in vitro* simulation will predict *in vivo* retinoid release needs to be investigated. The direct sponge–cervix contact achieved *in vivo* may impart a more rapid transfer of unbound or "free" retinoids to cervical tissue than anticipated from this study.

Both *c*-RA and *t*-RA, if protected from light, are stable for prolonged periods. Frolik et al.[17] found either compound to be stable for over 9 weeks when frozen. Hixson and Denine[8] found 0.001% dispersed solutions of these compounds to be stable for at least 72 h if protected from light. This is consistent with the results of the present study; *t*-RA and *c*-RA were stable at least 48 h at 37°C although there was slight photoisomerization of *t*-RA to *c*-RA. This was probably due to unavoidable exposure of the retinoid to light during the times of sampling and injection into the HPLC. This also concurs with the observations of Puglisi and De Silva.[18]

A final major finding of this study was that up to 50–70% of a retinoid solution was bound onto a collagen sponge matrix. This corroborates the drug sorption studies of Moorhatch and Chiou[15,19] in which there was 75–78% loss of retinol acetate to standard poly(vinyl chloride)(PVC) infusion bags over 24 h. Similarly, maximal binding was not achieved in this study until 24 h. This observation and the low recovery on methanol extraction suggest that the majority of the retinoid–sponge binding was due to direct penetration of the retinoids into the collagen matrix (absorption) rather than to adsorption which involves immediate but reversible binding to a surface monolayer, in this case, collagen. This is not surprising in light of one of the known physiologic functions of collagen in the body, namely its action as an ion exchanger, binding electrolytes, drugs, and metabolites. Such collagen–drug binding has previously been demonstrated for tetracycline, hemogentisic acid, antirheumatic agents, L-asparaginase,[20] and for various metals including zinc.[10] Such binding could adequately explain the incomplete recovery of retinoid over the course of this study. In this regard, Howard et al.[21] have recently documented two episodes of Vitamin A deficiency due to a loss of up to 60% of the retinol added to parenteral nutrition solutions stored in poly(vinyl chloride) infusion bags.

The collagen sponge insert as part of a vaginal-drug delivery device does not appear to be an effective slow release system for retinoids. This is due to rapid initial release of retinoids from the sponge in a simulated intravaginal environment and a time and concentration-dependent binding of all-*trans*-etinoic acid to the collagen matrix. These findings suggest that other systems and biomaterials must be developed intravaginal in retinoid delivery in human trials.

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