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Oestradiol Release from Self-setting Apatitic Bone Cement Responsive to Plasma-calcium Level in Ovariectomized Rats, and its Physicochemical Mechanism

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Abstract

The effect of plasma calcium levels on the release of oestradiol from a self-setting apatite bone cement containing 0·5% oestradiol was investigated in ovariectomized rats.

The profiles of in-vitro release from the cements in simulated body fluid containing 0, 5 or 10 mg calcium per 100 mL indicated that the rate of release of oestradiol decreased with increasing calcium concentration in the dissolution media. After subcutaneous implantation of oestradiol-loaded cement in healthy and vitamin D-deficient rats, oestradiol release in diseased rats with low plasma calcium levels was significantly higher than that in healthy rats.

These results suggest that in-vitro release of oestradiol from apatite bone cement was dependent on the calcium concentration in the buffer and that the in-vivo release of oestradiol from apatite bone cement was dependent on plasma calcium levels.

Because bone resorption and formation occur repeatedly in the functional units (called bone multicellular units) which maintain the dynamic equilibrium of mineral density in normal bone (Baron et al 1987) uncoupling between bone resorption by osteoclasts and bone formation by osteoblasts causes an absolute reduction in the amount of bone in osteoporosis. Osteoporotic fractures are observed more commonly among post-menopausal women, because bone mechanical strength is closely related to bone mineral density (Lee et al 1981) which reflects nutritional factors such as calcium (Charpuy et al 1992) and vitamin D (Dawson-Hughes et al 1990). Because steroid hormones with oestrogenic activity such as oestradiol and oestrone are considered to be related to the regulation of bone resorption and bone formation (Mclean et al 1968; Eriksen & Moskilde 1990), bone re-modelling after menopause becomes relatively inactive because of a decrease in the amount of hormone with the subsequent decrease in the mineral density of the bone. Therefore, these steroid hormones are used to prevent osteoporosis. In addition, according to the National Nutrition survey, the recommended daily dietary intake of calcium (600 mg) has not been achieved in Japan over the past decade (Okano et al 1993). Therefore, calcium deficiency is another important factor in osteoporosis in Japan (Okano et al 1993). Okano et al (1993) reported that the bioavailability of calcium products was also closely related to bone mineral density in vitamin D-deficient rats, because these had lower plasma-calcium concentrations and lower bone-mineral density.

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We have previously designed a delivery system for several drugs (Otsuka et al 1995) using a biocompatible self-setting calcium phosphate cement (Brown & Chow 1986; Corltz et al 1995) which is transformed into hydroxyapatite in the body. In another study (Otsuka et al 1997) the relationship between in-vitro and in-vivo release of indomethacin from these cements in healthy rats suggested that in-vivo indomethacin release was inhibited and slower than that observed in-vitro, reflecting the biological action in-vivo. We applied this finding to the drug delivery system and briefly reported (Otsuka et al 1996) that oestradiol release in vitamin D-deficient ovariectomized rats (Mclean et al 1968; Eriksen & Moskilde 1990) was greater than that in healthy rats after subcutaneous implantation of oestradiol-loaded cement. In this study, therefore, we have investigated the dependence of plasma calcium level on in-vitro and in-vivo release of oestradiol from apatite cement.

Materials and Methods

Self-setting apatite cement

The 0·5% β-oestradiol-loaded apatite cement consisting of an equimolar mixture of tetracalcium phosphate (TECP, $Ca_4(PO_4)_2O$) and dicalcium phosphate dihydrate (DCPD, $CaHPO_4 \cdot 2H_2O$) was prepared according to the procedure described previously (Otsuka et al 1997). Calcium phosphate cement powders (0·50 g) were mixed with phosphoric acid (25 mM; 0·20 mL) for 1 min to form a paste; the drug powders (2·5 mg) were then mixed homogeneously with this paste. The final paste was poured into a 15-mm-diameter plastic mould and stored at 37°C and 100% relative humidity for 24 h. The resulting hardened cement pellets were removed from the mould and embedded in silicon rubber, so that only one face of the pellet surface (1·77 cm^2) was exposed. The total weight of the cement pellets was 480 ± 5 mg.

X-ray diffraction

X-ray powder diffraction profiles of the cement and drug-loaded cement samples were acquired by powder X-ray diffraction analysis (XD-3A; Shimadzu, Japan; Cu radiation, 15 mA, 35 kV).

Fourier-transform infrared (FTIR) spectroscopy

The sample was dispersed in micronized KBr powder (sample concentration 5%) by means of a pestle and mortar. The mixed sample powder was loaded on a sample cup as a flat, loose powder bed with a flat surface formed by means of a micro spatula. FTIR spectra were obtained by powder diffuse reflectance on a Perkin-Elmer (Yokahama, Japan) type 1600 FTIR spectrophotometer; 50 co-added scans were collected at 4 cm^{-1} resolution and corrected using the Kubelka-Munk equation.

In-vitro drug-release test

The dissolution medium was simulated body fluid (SBF) at pH 7·25 comprising 142 mM Na^+, 5·0 mM K^+, 1·5 mM Mg^{2+}, 147·8 mM Cl^-, 2·5 mM Ca^{2+}, 4·2 mM HCO_3^-, 0·5 mM SO_4^{2-}, 1·0 mM HPO_4^{2+} and 1·0 g 4% bovine albumin (Kokubo et al 1991). Sample cement pellets and 25 mL SBF containing 10 mg/100 mL Ca^{2+}, SBF containing 5 mg/100 mL Ca^{2+} or Ca^{2+}-free SBF were introduced individually into 50-mL test tubes with caps. Each tube was fixed on sample holder in a thermostatically regulated water bath maintained at 37·0 ± 0·1°C, and shaken at 90 rev min^{-1}. During the release tests, the entire dissolution medium was replaced with fresh buffer at various intervals. All drug concentrations were determined by HPLC. The in-vitro data shown represent the averages of three measurements. In some experiments the medium was changed at 6-day intervals to assess the effect of Ca^{2+} concentration on oestradiol release rate.

Animal experiments

Animal care was conducted under the Animal Care Committee guidelines of Kobe Pharmaceutical University. Vitamin D-deficient female Wistar rats, 150–170 g (average weight 160 g), approximately 5 weeks old were used in the study. The rats were ovariectomized and then housed three per cage and fed a vitamin D- and calcium-deficient diet (Diet 11; Suda et al 1970) for 5 weeks. The bone-mineral density of the lumbar vertebrae of diseased and healthy rats was measured by bone mineral densitometry (model DCS-600R; Aroka) and found to be 41·8 ± 2·0 and 105·2 ± 6·5 mg cm^{-2}, respectively. Plasma calcium levels of the diseased rats averaged 5·1 mg/100 mL.

Under anaesthesia induced by intraperitoneal administration of sodium pentobarbital (45 mg kg^{-1}) a drug-loaded cement device containing 2·5 mg oestradiol was implanted in the subcutaneous tissue of the backs of the diseased rats. During the (2-week) drug-release test some of the diseased model rats were fed Diet 11 whereas other diseased rats, the disease-recovery-model rats, were fed a normal diet containing 1·1% (w/w) calcium. As a control the drug-loaded cement was also implanted on the back of a group of healthy rats which were fed a normal diet.

In a separate experiment, aqueous oestradiol solution (0·5 mL containing 5 mg oestradiol, 45 mL 1 N Na_2CO_3 and 5 mL Tween) was subcutaneously injected into the backs of the healthy rats, resulting in a dose of 333 $\mu g\ kg^{-1}$.

In both experiments, blood samples were collected at suitable time intervals from the tail artery. All experiments were performed on three rats.

High-performance liquid chromatography (HPLC)

Oestradiol was analysed by means of an HPLC system comprising Waters Associates model 510 solvent-delivery module, model 710B automatic injector, model 484 tuneable variable-wavelength UV absorbance detector (operated at 230 nm) and a Shimadzu CR-4A integrator. The pre-packed column (YMC Pakc ODS-AQ AQ-302, 150 × 4·6 mm i.d. × 5 μm particle size) was operated at 30°C and a mobile phase flow rate of 0·7 mL min^{-1}. The mobile phase was 50 : 46 : 4 water–acetonitrile–tetrahydrofuran.

Procedure for obtaining samples from in-vitro experiments. HCl (1 M; 1 mL) and diethyl ether (5 mL) containing oestriol (10 $\mu g\ mL^{-1}$) as internal standard were added to the sample (4·0 mL). The mixture was shaken for 15 min, centrifuged at 3000 rev min^{-1} for 15 min and the organic phase was decanted. Saturated aqueous $NaHCO_3$ solution (1 mL) was added to the organic phase and the mixture was shaken for 15 min, centrifuged at 3000 rev min^{-1} for 15 min, and the organic phase was decanted and dried by rotary evaporation at 37°C. HPLC mobile phase (200 μL) containing the internal standard was then added to the residue and mixed; 10 μL was then analysed by HPLC to determine the concentration of oestradiol.

Radioimmunoassay of oestradiol

Plasma oestradiol from in-vivo experiments was measured by radioimmunoassay using an oestradiol radioimmunoassay kit (Diagnostic Products, Los Angeles, CA).

Analysis of oestradiol pharmacokinetic data

The disposition of oestradiol in rats after subcutaneous injection as a single bolus into the back could be approximated by a biexponential model as shown in equation 2. The area under the curve (AUC), the maximum plasma drug concentration (C_{max}), time (T_{max}) required to reach C_{max} and the half life ($t_{1/2}$) were determined by non-linear least-squares analysis. The data were calculated on the basis of an average of three measurements from independent experiments. The relationship between the drug plasma level profile after subcutaneous administration of cement (C_p^c) and the rate of input $C_p^{sc}(t)$ (drug plasma level profile after subcutaneous administration of solution) for a drug with linear pharmacokinetics is given by (Yamaoka & Tanigawura 1983):

$$C_p^c = f_a(t)C_p^{sc}(t) = \frac{D_c}{D_{sc}}\int_0^t f_a(\tau)C_p^{sc}(t-\tau)d\tau \qquad (1)$$

where

$$f_a(t) = A\exp(-\alpha t) + B\exp(-\beta t) \qquad (2)$$

D_c and D_{sc} are doses administered via cement and subcutaneous injection, respectively, A, B, α and β are constants, t and τ are the time and time interval for calculation, respectively, and $f_a(t)$ is the unit impulse response, i.e., the response to an instantaneous unit dose at the input site.

The in-vivo oestradiol release profiles were calculated using the deconvolution computer program PROGRAM 4-4

(Yamaoka et al 1981; Yamaoka & Tanigawara 1983) on the basis of eqn 1.

Plasma calcium measurements

The plasma calcium concentration was determined by the method described by Kitano & Ueda (1971). Briefly, the blue complex formed between methylxylenol and calcium ions was measured spectrophotometrically (UV 160A; Shimadzu, Kyoto Japan) at 610 nm.

Results and Discussion

Characterization of self-setting calcium phosphate cement containing oestradiol

Fig. 1 shows the X-ray diffraction profile of self-setting apatite cement loaded with 0·5% oestradiol. The fresh fixed cement showed a diffraction pattern typical of hydroxyapatite with diffraction peaks at $2\theta = 29{\cdot}5°$ from TECP, indicating that the metastable calcium phosphates, DCPD and TECP had been transformed into hydroxyapatite. Because the hydroxyapatite diffraction peaks of the cement were much broader than those of synthetic hydroxyapatite, the cement was transformed into apatite with a low crystallinity during cement preparation.

Fig. 2 shows the FTIR spectrum of fixed cement containing 0·5% oestradiol; this again indicates absorption peaks of a typical hydroxyapatite. These results suggested that mixing the cement paste with oestradiol did not interfere with the fixing process in the formation of apatite in the cement.

Effect of calcium level on in-vitro oestradiol release from apatite cement

Fig. 3 shows the profile of in-vitro release of oestradiol from 0·5% oestradiol-loaded apatite cement systems into SBF containing 0, 5 or 10 mg calcium $(100\ mL)^{-1}$. The in-vitro release of oestradiol from cement systems into SBF containing 0, 5 or 10 mg calcium/100 mL was 410, 346 and 241 μg, respectively, after 24 days, indicating that the rate of release of oestradiol from the apatite cement decreased with increasing calcium concentration in the dissolution media.

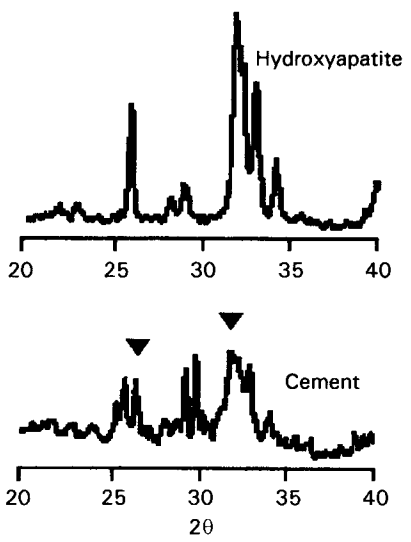

FIG. 1. X-ray diffraction profiles of apatitic cement containing oestradiol and synthetic hydroxyapatite. The peaks indicated are evidence that the cement is apatitic.

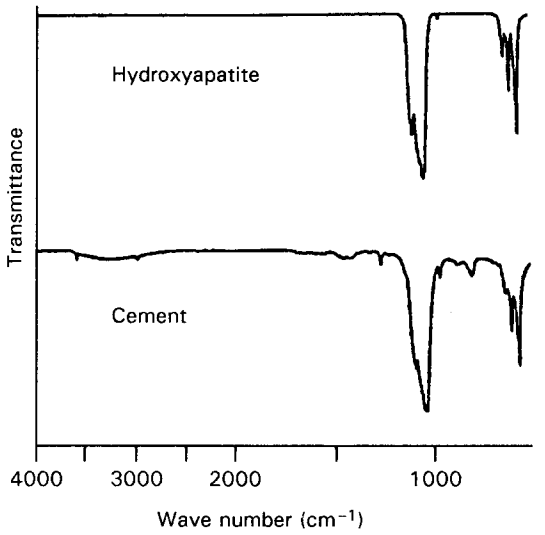

FIG. 2. FTIR of apatitic cement containing oestradiol and synthetic hydroxyapatite.

The rate-limiting step of the release of the drug from the matrix was basically the drug diffusion process in the micro pores of the matrix. Drug release from the planar surface of homogeneous drug-loaded matrix systems follows the Higuchi equation (Higuchi 1963):

$$M_t = \sqrt{C_s \frac{D_i \varepsilon}{\tau}(2C_d - \varepsilon C_s)t} \qquad (3)$$

where M_t is the amount of drug released per unit area after time t, D is the diffusion coefficient of the drug, C_s is the solubility, C_d is the concentration of drug in the matrix, τ is the tortuosity and ε is the porosity.

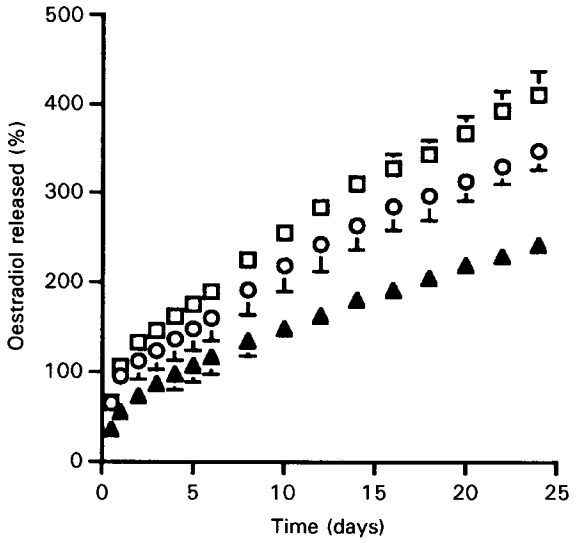

FIG. 3. In-vitro release of oestradiol from oestradiol-loaded apatitic cements in simulated body fluid (pH 7·25) containing 0 (□), 5 (○) and 10 mg (▲) calcium/100 mL at 37°C (n = 3).

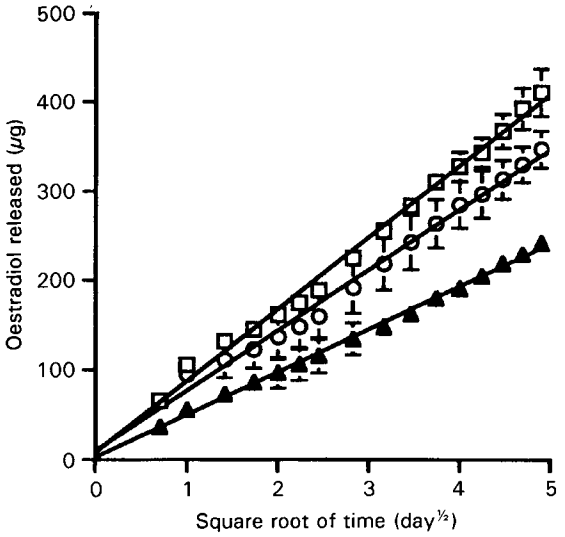

FIG. 4. Plots of in-vitro release of oestradiol from the apatitic cement in simulated body fluid containing different levels of calcium, against the square root of time. □ Calcium-free simulated body fluid, ○ with 5 mg calcium/100 mL, ▲ with 10 mg calcium/100 mL (n = 3).

Therefore, the profiles of in-vitro release of oestradiol from apatite cements in SBF containing different levels of calcium were used to plot the release against the square root of time, as shown in Fig. 4. The in-vitro profiles of oestradiol release from oestradiol-loaded cements were linear on the Higuchi plot, but the slope decreased with increasing calcium concentrations in the buffer. Table 1 shows the rate constants for in-vitro release of oestradiol, evaluated from the slope of the plots by the least-squares method. The rate constant for in-vitro release of oestradiol from the cement in SBF containing 10 mg calcium/100 mL was significantly lower than in that containing 5 mg/100 mL or that containing no calcium.

As reported previously (Otsuka et al 1994a), release of indomethacin from apatite cement systems in phosphate buffer follows the Higuchi equation. However, indomethacin release from apatitic cements (Otsuka et al 1994b) in SBF with calcium and phosphate ions in supersaturated solution, such as that in body fluid, did not follow the theoretical mechanism. In the current study, the results of in-vitro oestradiol release from apatite cement also depended on the concentration of calcium in the SBF.

To clarify the dependence on calcium concentration of drug release from apatite cement, drug release rates were measured after change of the dissolution medium during the in-vitro oestradiol release test. Fig. 5 and Table 2 shows the effect of calcium concentration on in-vitro oestradiol release from apatite cement. When the solution was changed to calcium-free SBF, the rate of release of oestradiol increased significantly, but when it was changed to SBF containing 10 mg $(100\ mL)^{-1}$ calcium, the rate decreased. Measurement of the in-vitro release of oestradiol in SBF containing supersaturated calcium concentrations also suggested that changes in the geometrical structure of the pores of the apatite cement depend on the concentration of calcium in SBF, owing to precipitation of hydroxyapatite on the cement surface during the drug-release test as a result of the extent of supersaturation of SBF with regard to the solubility of hydroxyapatite. Conversely, in SBF unsaturated with calcium, the hydroxyapatite in the cement matrix dissolved or eroded, or both, accelerating drug-release. Therefore, in-vitro release of oestradiol from the cement matrix reflected a decrease or increase of the effective diffusivity of oestradiol in the pores, indicating that the release rates depended on calcium levels in SBF; this explains the calcium-dependence of drug release from the matrix.

Table 1. Effect of calcium concentration on the in-vitro rate of release of oestradiol from apatite cement.

Calcium concentration (mg/100 mL)	Drug-release constant ($\mu g\ day^{-½}$) ± s.d.*
10	47·7 ± 1·0†
5	67·3 ± 2·1
0	80·00 ± 9·97

*Standard deviation (n = 3). †$P < 0.05$ compared with 0 and 5 mg calcium/100 mL (Student's t-test).

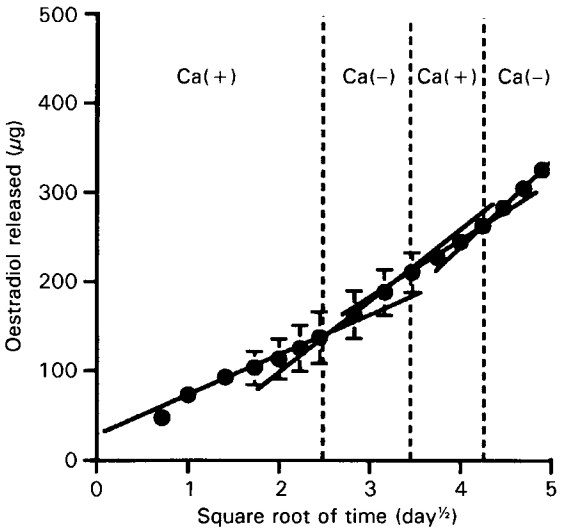

FIG. 5. The effect of calcium concentration in simulated body fluid on in-vitro oestradiol release from the apatitic cement. Ca− calcium-free, Ca+ with 10 mg calcium/100 mL (n = 3).

Table 2. Dependence on calcium concentration of the in-vitro release of oestradiol from apatite cement.

Sampling time (days)	Calcium concentration (mg/100 mL)	Drug-release constant ($\mu g\ day^{-½}$) ± s.d.*
0–6	10	46·1 ± 12·6†
6–12	0	72·1 ± 6·2
12–18	10	61·7 ± 11·9‡
18–24	0	95·9 ± 7·1

*Standard deviation (n = 3). †$P < 0.05$, significantly different from result for 6–12 days; ‡$P < 0.05$ compared with result for 18–24 days (Student's t-test).

Pharmacokinetics of oestradiol-loaded apatite cement in healthy and diseased rats

Fig. 6 shows the plasma calcium concentration profiles after subcutaneous implantation of oestradiol-loaded cement in healthy and diseased rats. Healthy rats have a constant plasma calcium concentration of approximately 10 mg/100 mL.

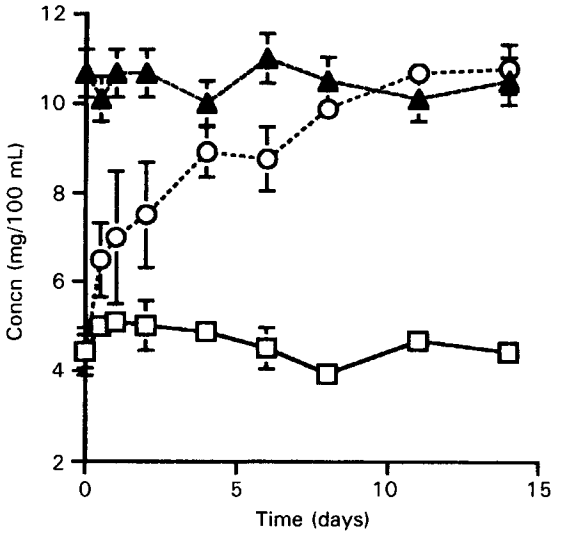

FIG. 6. Plasma calcium levels of diseased and healthy rats after subcutaneous administration of oestradiol-loaded apatitic cements. □ Diseased rats, ○ recovery model, ▲ healthy rats (n = 3).

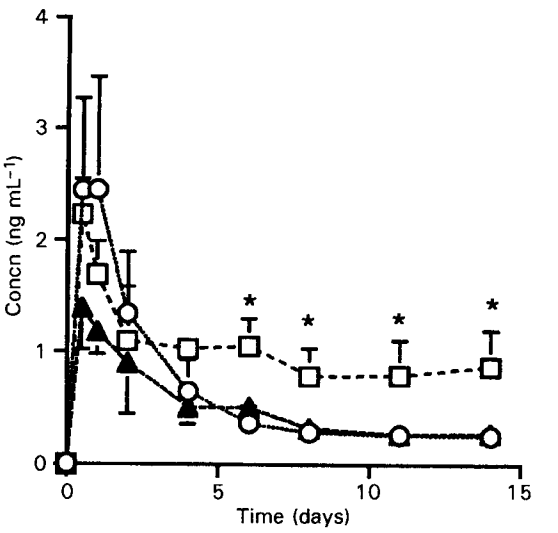

FIG. 7. Plasma oestradiol levels of diseased and healthy rats after subcutaneous administration of oestradiol-loaded apatite cements. □ Diseased rats, ○ recovery model, ▲ healthy rats. *$P < 0.05$ compared with healthy rats (n = 3).

However, the plasma calcium level of diseased rats was lower, approximately 5 mg calcium/100 mL; this level was maintained during drug release. In contrast, the plasma calcium level of the recovery-model rats was increased when they were fed calcium, and reached the healthy level of approximately 10 mg/100 mL after 7 days.

Fig. 7 shows plasma oestradiol concentrations in diseased and healthy rats after subcutaneous implantation of oestradiol-loaded cement. The plasma oestradiol concentrations in the diseased rats increased rapidly, attaining a maximum level (C_{max}) of 2·23 ng mL^{-1}. Plasma oestradiol concentrations in the diseased rats decreased gradually, yet after 5 days were still significantly higher than those in the healthy group, with the release continuing for over 2 weeks. In the recovery-model rats, the plasma oestradiol concentrations increased rapidly, attaining a maximum level (C_{max}) of 2·55 ng mL^{-1}. These levels, however, decreased gradually, and after 5 days were almost the same as those of healthy rats. In contrast, the C_{max} for the healthy rats (1·43 ng mL^{-1}) was much lower than that for the diseased rats and recovery-model rats, and the plasma levels after 5 days were significantly lower than those of the diseased rats.

Table 3 shows the comparative bioavailability of oestradiol-loaded apatite cement. The bioavailability of the cement in the diseased rats was significantly higher than in the healthy and recovery-model rats, but was lower than that measured after subcutaneous administration. Because the percentages of drug remaining in the cements of the healthy, recovery-model and diseased rats were 79·7 ± 2·1, 75·88 ± 1·6 and 74·1 ± 1·2%, respectively, the bioavailability of the cements was lower than after subcutaneous administration.

Relationship between the plasma calcium level and oestradiol release in healthy and diseased rats

Fig. 8 shows the in-vivo rates of drug release obtained by deconvolution for diseased and healthy rats. The results suggest that the in-vivo rate of release of oestradiol in the healthy rats was much lower than in the diseased rats, and that the rate of release of the recovery-model rats decreased with time.

Fig. 9 shows plots of the in-vivo amounts of drug released, obtained by deconvolution for diseased and healthy rats, against the square root of time. The results suggest that the in-vivo rate of oestradiol release in the healthy rats was much lower than in the diseased rats, and that the rate of drug release in the recovery-model rats decreased with time. To clarify drug release in diseased and healthy rats, it was assumed that drug release followed the Higuchi equation. Drug-release rate constants evaluated the initial (0–4 days) and later (4–14 days)

Table 3. Bioavailability of oestradiol from loaded apatite cements in diseased and healthy rats.

Animal model	Healthy	Recovery	Diseased	Injected subcutaneously
Dose	15·0 ± 0·4	15·0 ± 0·4	15·0 ± 0·3	0·330 ± 0·002‡
Amount released in 14 days during in-vivo release test	2·95 ± 0·14	3·23 ± 0·18	3·48 ± 0·16	0·333 ± 0·002
Area under plasma concentration–time curve (μg day mL^{-1}) ± s.d.*	6·62 ± 1·11	9·00 ± 3·03	14·05 ± 3·55	2·31 ± 0·53
Comparative area under the curve (%)† ± s.d.*	30·6 ± 6·4	35·4 ± 12·9	66·4 ± 15·6	100·0 ± 23·1

*Standard deviation (n = 3). †Calculated from cAUC = area under plasma concentration–time curve/(area under plasma concentration–time curve for subcutaneous injection × amount released in 14 days during in-vivo release test). ‡$P < 0.05$ compared with result for diseased rats (Student's *t*-test).

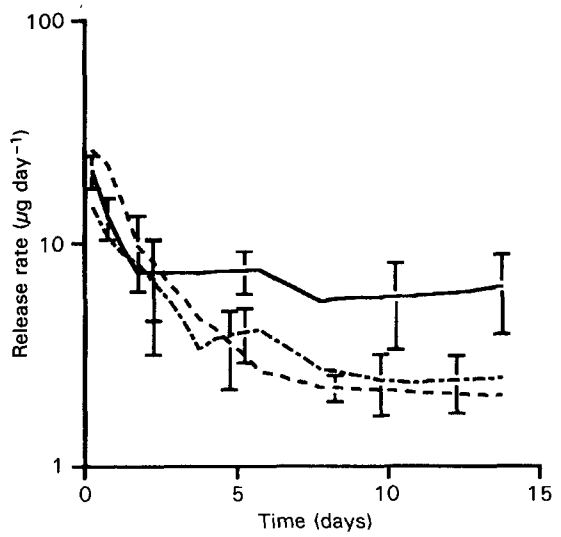

FIG. 8. In-vivo rates of release of oestradiol from loaded apatite cement in healthy and diseased rats. (—) Diseased rats, (- - -) recovery model, (– – – -) healthy rats (n = 3).

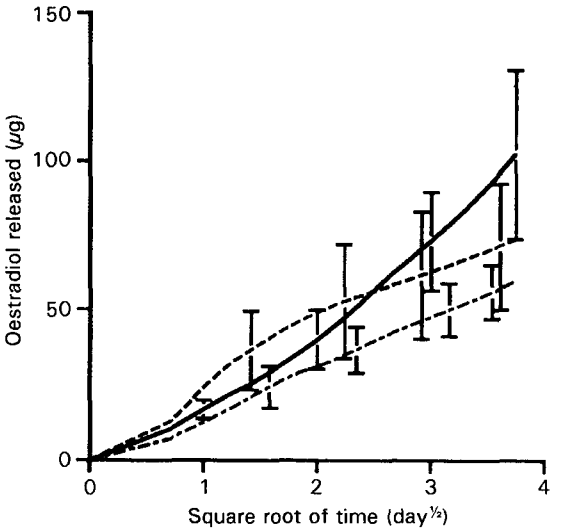

FIG. 9. Plots of the amount of drug released in-vivo from oestradiol-loaded apatite cement in diseased and healthy rats, against the gesquare root of time. (—) Diseased rats, (- - -) recovery model, (– – – -) healthy rats (n = 3).

stages by the least-squares method, and are summarized in Table 4. The later release-rate constants of healthy and recovery-model rats were significantly lower from those of the diseased rats, but initial release rate constants were not significantly different. The later release-rate constant for the recovery model was significantly lower than that for initial release.

The results for in-vivo drug release suggested that the plasma oestradiol concentration for healthy rats was much lower than that for diseased rats—in healthy rats the plasma calcium concentration was twice that of diseased rats, and the drug concentrations in the diseased rats in the later dissolution tests were dependent on plasma calcium levels. The in-vivo rate of release of oestradiol in the initial stages in the recovery-model rat was not significantly different from that for the diseased rats ($P > 0.05$); it was, however, significantly lower ($P < 0.05$) in the later stage. Because the results for in-vivo release of oestradiol were consistent with in-vitro drug release data, these results supported the hypothesis that drug release from the cement matrix composed of hydroxyapatite crystals was governed by demineralization and re-mineralization of calcium phosphate. This led to an increase in the tortuosity of the pores and a decrease in the porosity of the cement matrix during drug release; this in turn reduced the effective diffusivity (Higuchi 1963) of the drug in the pores and hence led to a decrease in the rate of drug release in healthy rats. In contrast, in hyposaturated solution, i.e. under conditions when the plasma calcium level was lower, the pore volume increased as a result of dissolution or erosion (or both) of hydroxyapatite at the cement surfaces. Therefore, the effective diffusivity of drug in the pores increased leading to an increase in the rate of drug release from the cement matrix. The in-vivo release of oestradiol for the recovery-model rats supported the hypothesis that the drug release behaviour of the apatitic cement drug-delivery system was responsive to plasma calcium levels.

These in-vitro and in-vivo results lead to the conclusion that apatite cement is an 'intelligent material' with the ability to change the rate of drug release appropriately in response to changes in plasma calcium concentration.

Table 4. In-vivo release of oestradiol from apatite cements in diseased and healthy rats.

Animal model	Sampling time (days)	Drug-release constant ($\mu g\ day^{-½}$) ± s.d.*
Healthy rats	0–4	18·9 ± 7·4
	4–14	16·2 ± 4·4†
Recovery	0–4	27·3 ± 10·4‡
	4–14	13·5 ± 1·6§
Diseased	0–4	22·4 ± 7·2
	4–14	35·1 ± 11·5

*Standard deviation (n = 3). †$P < 0.05$ compared with result after 4–14 days for diseased rats; ‡$P < 0.05$ compared with result after 4–14 days for recovery model; §$P < 0.01$ compared with result after 4–14 days for diseased model.

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