J. Pharm. Pharmacol. 1996, 48: 798–800
Received July 13, 1995
Accepted March 22, 1996

Physicochemical Characterization of Bisphosphonic Carboxyfluorescein for Osteotropic Drug Delivery

JIRO FUJISAKI, YUJI TOKUNAGA, TOSHIYA TAKAHASHI, SABURO MURATA, FUMIO SHIMOJO AND TAKEHISA HATA

Pharmaceutical and Pharmacokinetic Research Laboratories, Fujisawa Pharmaceutical Company Ltd, 1-6, 2-chome, Kashima, Yodogawa-ku, Osaka 532, Japan

Abstract

Disodium (fluorescein-6-carbonyloxy)acetoaminomethylene bisphosphonate (CF-BP), a prodrug of 6-carboxyfluorescein, is efficiently absorbed by the skeleton where it hydrolyses to carboxyfluorescein. An osteotropic drug-delivery system based on this bisphosphonic prodrug has been developed as a novel method for site-specific and controlled delivery of drugs to the bone. In this study the physicochemical properties of the prodrug have been characterized by investigating the affinity of CF-BP for hydroxyapatite and the hydrolysis of the compound to carboxyfluorescein.

In the binding study, CF-BP bound very rapidly to hydroxyapatite without degradation and carboxyfluorescein was subsequently gradually released by hydrolysis of bound CF-BP. Hydrolysis of CF-BP in buffer solutions followed pseudo-first-order kinetics, and quantitative regeneration of carboxyfluorescein was observed. In addition, regeneration of carboxyfluorescein from CF-BP was accelerated in the presence of fresh rat plasma.

These results suggest that CF-BP has the physicochemical properties required for site-specific and controlled delivery of carboxyfluorescein to bones.

Bone-targetted drug delivery has the potential to improve the therapy of skeletal diseases such as osteoporosis, osteoarthritis and bone metastasis. There are, however, many difficulties in the efficient delivery of drugs to bone by conventional means because bone tissue comprises mostly the inorganic compound hydroxyapatite (Eastoe 1956). We have recently proposed an osteotropic drug-delivery system based on a bisphosphonic prodrug concept as a novel method for site-specific and controlled delivery of drugs to the bone (Fujisaki et al 1995). To verify the concept, the bisphosphonic prodrug of carboxyfluorescein, disodium (fluorescein-6-carbonyloxy)acetoaminomethylene bisphosphonate (CF-BP; Fig. 1) was synthesized as a model compound, and in-vivo disposition after intravenous injection was investigated in rats. CF-BP was efficiently absorbed by the skeleton (62·1% of dose), most of the remainder being excreted in the urine (35·9% of dose). Regeneration of carboxyfluorescein by hydrolysis of CF-BP was, furthermore, observed in the bone. To achieve targetted drug delivery based on site-specific transport of prodrug, it is essential that the prodrug not only has high affinity for the targetted site but also appropriate lability in the site, ideally without regeneration in a non-target site.

In this study we have investigated both the affinity of CF-BP for hydroxyapatite and the in-vitro hydrolysis of CF-BP to clarify the basic physicochemical properties required of an osteotropic prodrug.

Materials and Methods

Materials

5,6-Carboxyfluorescein was purchased from Eastman-Kodak (Rochester, NY, USA) and 6-carboxyfluorescein was prepared by the charcoal/LH-20 procedure (Ralston et al 1981). Disodium (fluorescein-6-carbonyloxy)acetoaminomethylene bisphosphonate (CF-BP; Fig. 1) was synthesized as previously reported (Fujisaki et al 1995). Briefly, bromoacetic acid was added to tetraethylaminomethylene bisphosphonate, and carboxyfluorescein was then coupled by the reaction of alkyl halide and carboxylate to yield the carboxylic ester bond. The phosphonate ester in this intermediate was selectively hydrolysed to (fluorescein-6-carbonyloxy)acetoaminomethylene bisphosphonic acid with trimethylsilyl iodide in acetonitrile at − 20°C. CF-BP was then prepared as disodium salt with sodium acetate. The yields at each synthetic step were 70–82%. Hydroxyapatite with a molar Ca : P ratio of 2·03 was purchased from Seikagaku Kogyo (Tokyo, Japan). The particle size of the crystals was 50–100 μm. All other reagents and solvents were of analytical grade.

Hydrolysis study

Hydrolysis experiments were performed on CF-BP in buffer solution and rat plasma. The buffers used in the hydrolysis study were hydrochloric acid-sodium chloride (pH 1 and 2), phosphate (pH 3–8 in intervals of one pH unit) and carbonate (pH 9 and 10); buffer solutions (0·1 M) ranging from pH 1 to pH 9 were prepared and adjusted to an ionic strength of 0·2 with sodium chloride. Male Sprague–Dawley rats, 240–260 g,

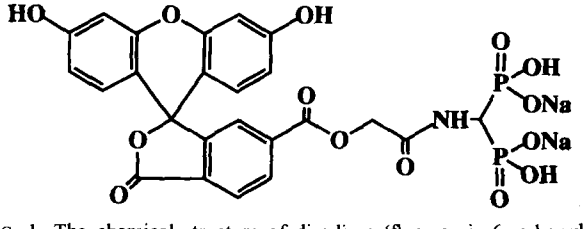

FIG. 1. The chemical structure of disodium (fluorescein-6-carbonyloxy)acetoaminomethylene bisphosphonate (CF-BP).

Correspondence: J. Fujisaki, Pharmaceutical and Pharmacokinetic Research Laboratories, Fujisawa Pharmaceutical Company Ltd, 1-6, 2-chome, Kashima, Yodogawa-ku, Osaka 532, Japan.

were used to obtain plasma. Preheated rat plasma was prepared by heating fresh plasma at 60°C for 1 h. All hydrolysis experiments were performed at 37 ± 0·20°C and initiated by the addition of a stock aqueous solution (200 μM) to pre-warmed media to give a final drug concentration of 10 μM. At appropriate times aliquots were withdrawn and stored at −20°C until assay. The samples were analysed directly by HPLC.

Binding study

All binding studies were performed with hydroxyapatite suspended in buffer solutions. Buffer solution containing 0·155 M KCl buffered at pH 7·4 with 0·01 M diethylbarbituric acid was equilibrated with hydroxyapatite to minimize dissolution of the crystals (Jung et al 1973). The binding experiment was performed after equilibration of 2 mg hydroxyapatite/9·5 mL of the buffer solution for 2 h at 37°C. Subsequently, 0·5 mL stock solution of CF-BP or carboxyfluorescein was added to the hydroxyapatite suspension to give a final drug concentration of 10 μM. At appropriate times portions of solution were withdrawn and filtered through a membrane filter (Samprep-LCR, pore size 0·5 μm; Millipore, Tokyo, Japan). After dissolution in distilled water, the sample solutions were analysed by HPLC.

HPLC assay

Carboxyfluorescein and CF-BP were assayed by reversed-phase gradient HPLC with fluorescence detection at an excitation wavelength of 495 nm and an emission wavelength of 525 nm (Fujisaki et al 1995). The mobile phase was a mixture of methanol and pH 7·4 citrate-phosphate buffer (10 mM) containing 10 mM tetrabutylammonium bromide as ion-pair reagent. The proportion of methanol in the mobile phase was increased linearly from 20 to 40% in 20 min followed by maintenance of this composition for 20 min; the retention times of carboxyfluorescein and CF-BP were 20·9 and 19·2 min, respectively.

Results and Discussion

Bisphosphonates are non-hydrolysable analogues of pyrophosphonate and bind to hydroxyapatite as a result of chemical adsorption of the bisphosphonic acid group; this accounts for their rapid uptake by bone (Jung et al 1973; Fleisch 1988). For osteotropic delivery via the bisphosphonic prodrug, it is necessary that the bisphosphonic prodrug not only has affinity for hydroxyapatite but is also hydrolysed to the parent drug. In this study, therefore, we investigated the hydrolysis of CF-BP and the affinity of CF-BP for hydroxyapatite.

The typical time courses of disappearance of CF-BP and appearance of carboxyfluorescein in buffer solutions are shown in Fig. 2. Regeneration of carboxyfluorescein may be a result of direct cleavage of the carboxylic ester linkage. The disappearance of CF-BP at each pH followed pseudo-first-order kinetics with respect to concentration. Table 1 summarizes the observed pseudo-first-order rate constants (k_{obs}) and half-lives (t½). CF-BP showed almost constant k_{obs} values between pH 1 and pH 5. Above pH 5, however, k_{obs} increased with increasing pH. As shown in Table 1, CF-BP was hydrolysed to carboxyfluorescein in fresh rat plasma with a half-life of 0·8 days, but no hydrolysis of CF-BP was observed in preheated plasma over a period of 6 h. These results suggest the susceptibility of CF-BP to enzymatic hydrolysis.

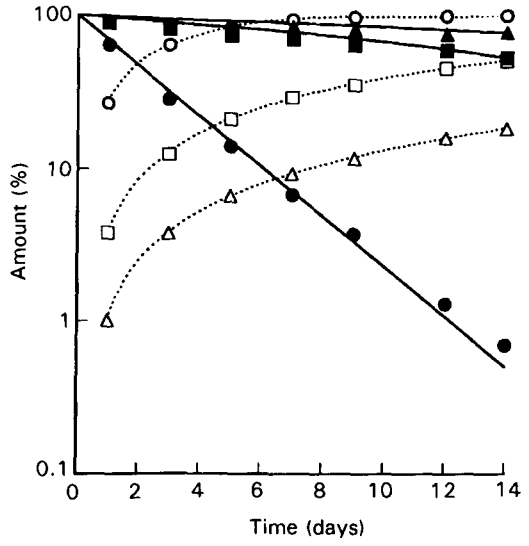

FIG. 2. Typical plots for appearance of carboxyfluorescein (–) and disappearance of CF-BP (···) in buffer solutions at 37°C. ▲, △ pH 1; ■, □ pH 6; ●, ○ pH 8.

Table 1. Pseudo-first-order rate constants (k_{obs}) and half-lives (t½) of CF-BP in buffer and rat plasma at 37°C[a].

Medium	k_{obs} (day^{-1})	t½ (days)
Buffer, pH 1	0·0333	20·8
Buffer, pH 2	0·0378	18·3
Buffer, pH 3	0·0513	13·5
Buffer, pH 4	0·0240	28·9
Buffer, pH 5	0·0230	30·1
Buffer, pH 6	0·0414	16·7
Buffer, pH 7	0·1101	6·3
Buffer, pH 8	0·3518	2·0
Buffer, pH 9	2·9570	0·2
Rat plasma	0·881	0·8

[a]CF-BP was stable for 6 h in rat plasma preheated at 60°C. The data presented are means of measurements in triplicate. CVs are less than 10%.

Fig. 3 shows the disappearance of CF-BP and carboxyfluorescein from the aqueous phase as a function of incubation time. Whereas the amount of carboxyfluorescein in the aqueous phase remained constant for 120 min, more than 90% of CF-BP disappeared from the aqueous phase during the first 30 min. These findings suggest that CF-BP binds to the calcium of hydroxyapatite as the bisphosphonic derivative. No CF-BP was detected in the aqueous phase 6 h after the addition (data not shown) indicating that all the CF-BP was adsorbed by the hydroxyapatite. The subsequent hydrolysis study was, therefore, performed using the same suspension. Fig. 4 shows the appearance of carboxyfluorescein in the aqueous phase as a function of incubation time. The amount of carboxyfluorescein observed in the aqueous phase gradually increased until 14 days after incubation. As the appearance of carboxyfluorescein in the aqueous phase was a result of the hydrolysis of CF-BP bound to hydroxyapatite, the residual amount of CF-BP bound to hydroxyapatite was calculated by subtraction of the amount

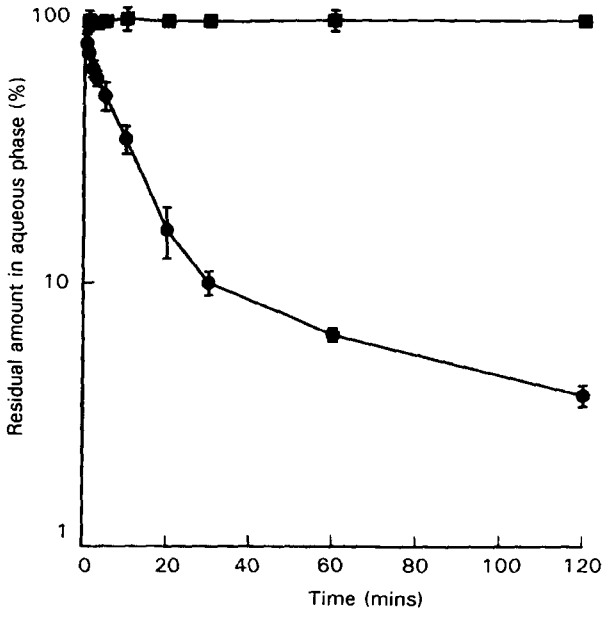

FIG. 3. Disappearance of CF-BP (●) and carboxyfluorescein from the aqueous phase at 37°C.

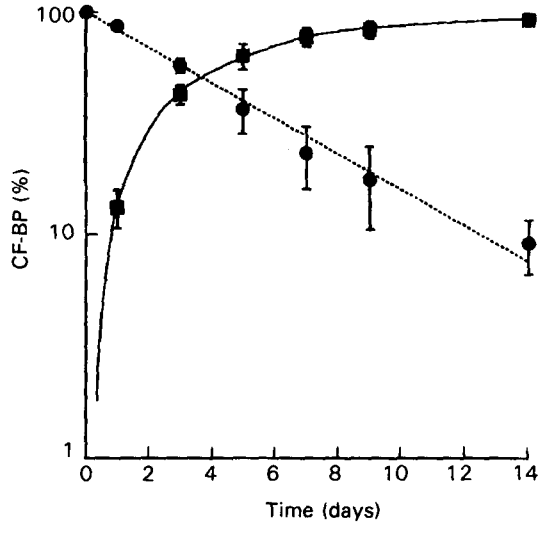

FIG. 4. Appearance of carboxyfluorescein (■) regenerated from CF-BP (●) bound to hydroxyapatite in buffer solution.

of carboxyfluorescein released from the amount of CF-BP added. The calculated amount of CF-BP bound to hydroxyapatite is also plotted as a function of time in Fig. 4. The residual amount of CF-BP bound to hydroxyapatite decreased monoexponentially for 14 days with a half-life of 3·8 days. CF-BP was not, on the other hand, detected in the aqueous phase of the hydroxyapatite suspension during the 14 days. Judging from these results, CF-BP bound rapidly to hydroxyapatite without degradation and thereafter carboxyfluorescein was gradually released as a result of the hydrolysis of CF-BP bound to hydroxyapatite. When the bisphosphonic prodrug is administered systemically, the bone-concentration-time profile of the parent drug is determined by at least four factors: uptake of the bisphosphonic prodrug into the bone; retention of the bisphosphonic prodrug by the bone; hydrolysis of the bisphosphonic prodrug in the bone; retention of the parent drug by the bone. Of these factors, either the first or second applies to the bisphosphonic prodrug, i.e. bisphosphonates are rapidly distributed in bone and retained for a long period of time depending on the turnover of the bone itself (Fleisch 1988). The fourth factor may also apply to the bisphosphonic prodrug. We suggest that the diffusion-limited release of the parent drug regenerated in the bone; carboxyfluorescein regenerated in the bone was retained by the bone irrespective of the low affinity of carboxyfluorescein for hydroxyapatite. Otsuka et al (1994) reported the sustained release of indomethacin from calcium phosphate cement matrix. As a result, the third factor, the hydrolysis of bisphosphonic prodrug in the bone, is considered to be the most important factor determining the concentration-time pattern of the parent drug in the bone; the structure of the spacer used for the linkage between the parent drug and bisphosphonic group may considerably influence the bone-concentration-time profile of the parent drug.

We conclude that the binding of bisphosphonic prodrug to hydroxyapatite and the subsequent release of the parent drug from hydroxyapatite represent uptake by the bone and hydrolysis in the bone, respectively. They might, therefore, be useful indicators for the rational design of an osteotropic drug-delivery system.

References

Eastoe, J. E. (1956) The organic matrix of bone. In: Bourne, G. H. (ed.) The Biochemistry and Physiology of Bone. Academic Press, New York, pp 81–105

Fleisch, H. (1988) Bisphosphonates: a new class of drugs in diseases of bone and calcium metabolism. In: Baker, P. F. (ed.) Handbook of Experimental Pharmacology, vol. 83, Springer, Berlin, pp 441–466

Fujisaki, J., Tokunaga, Y., Takahashi, T., Hirose, T., Shimojo, F., Kagayama, A., Hata, T. (1995) Osteotropic drug delivery system (ODDS) based on bisphosphonic prodrug. I: Synthesis and in vivo characterization of osteotropic carboxyfluorescein. J. Drug Targeting 3: 273–282

Jung, A., Bisaz, S., Fleisch, H. (1973) The binding of pyrophosphonate and two diphosphonates by hydroxyapatite crystals. Calcif. Tissue Res. 11: 269–280

Otsuka, M., Matsuda, Y., Suwa, Y., Fox, J., Higuchi, W. (1994) A Novel skeletal drug delivery system using self-setting calcium phosphate cement. 2. Physicochemical properties and drug release rate of the cement-containing indomethacin. J. Pharm. Sci. 83: 611–615

Ralston, E., Hjelmeland, L. M., Klausner, R. D., Weinstein, J. N., Blumenthal, R. (1981) Carboxyfluorescein as a probe for liposome-cell interactions: effect of impurities, and purification of the dye. Biochem. Biophys. Acta 649: 133–137