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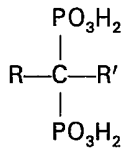
(54) **Pharmaceutical compositions containing biphosphonic acids**

(57) Biphosphonic acids of general formula I:



(I)

in which R is a fluorine atom or a linear or branched alkyl radical containing from 1 and 5 carbon atoms, which may also be substituted by one or more amino groups or fluorine atoms or both amino groups and fluorine atoms, R′ is hydroxy or fluorine, and their salts with an alkali metal, an organic base or a basic amino-acid, exhibit valuable properties in the treatment of urolithiasis or in the treatment as inhibitors of bone reabsorption.

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SPECIFICATION

Pharmacologically active biphosphonates, process for the preparation thereof and pharmaceutical compositions thereform

The present invention relates to the preparation of biphosphonic acids and their salts. The invention also relates to pharmaceutical compositions suitable for the treatment of urolithiasis and capable of inhibiting the bone reabsorption.

It is known that condensed phosphates in low concentrations may prevent the deposition of calcium carbonate from solutions; in addition to this effect, the condensed phosphates, and among them the pyrophosphates, are capable of inhibiting the precipitation of calcium phosphate when they are added even in low concentrations to solutions of calcium phosphates. This inhibitory action manifests itself both in the absence as well as in the presence of crystals of apatite.

In addition, the condensed phosphates retard the transformation of calcium phosphate from the amorphous phase to the crystalline phase without, however, influencing the formation of the amorphous phase. The marked effect *in vitro* of pyrophosphate (PP) on calcium phosphate in concentrations close to the concentrations found in biological fluids, has suggested that pyrophosphate may protect soft tissues from mineralization. In bone, the pyrophosphate (PP) could also regulate the progress of calcification and, therefore, influence the transformation of calcium and phosphate. The PP in bone which has already been mineralized influences the movement of calcium and phosphate towards the interior and the exterior of the bone. In spite of all the knowledge which has been acquired with respect to PP, its therapeutic use has proved impossible because of the rapid hydrolysis which the substance undergoes, both when it is administered by the oral route as well as when it is administered by the systemic route.

In view of the great interest connected with PP, investigations have been carried out for the purpose of making substances with similar activity but resistant to hydrolysis. This object has been achieved partially with the synthesis of biphosphonates, that is substances which contain the group P—C—P instead of the group P—O—P. The action of the biphosphonates on calcium salts is similar to the action of PP; indeed, even in low concentration, they exhibit the following actions:

they inhibit the precipitation of calcium phosphate from solutions;

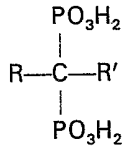
they block the transformation of amorphous calcium phosphate into the crystalline form without, however, inhibiting the formation of the initial phase;

they block the aggregation of crystals of hydroxyapatite;

they retard the degree of dissolution of crystals of hydroxyapatite after the latter have absorbed the biphosphonates from the solutions.

Several pharmacological and clinical studies in the scientific literature, however demonstrate that, in spite of certain analogies in activity, the several biphosphonates used up to the present time in the treatment of osteopthia exhibit some quite serious drawbacks with respect to the degree of toxicity in animals and the tolerability or the inducement of negative colateral side effects in men.

It has been now found surprisingly that some biphosphonic acids of general formula I:



(I)

in which R is fluorine or a linear or branched alkyl residue containing from 1 to 5 carbon atoms, which may optionally be substituted by one or more substituents such as amino groups and/or fluorine atoms and R′ is hydroxy or fluorine, and their salts with alkali metals, organic bases and basic aminoacids, are very suitable for the treatment of urolithiasis and as inhibitors of the bone reabsorption because they exhibit high activity which is not accompanied by the side effects hereinabove with respect to pyrophosphate (PP).

Several biphosphonic acids have been described in the literature. In particular biphosphonic acids of general formula I in which R is an unsubstituted alkyl and R′ is hydroxy may be prepared by reacting an acyl halide or the anhydride of an acid with phosphorous acid or phosphorous trichloride. This procedure, although it gives a good yield in the case in which R is ethyl, is less suitable for the achievement of analogues containing an alkyl residue with a higher molecular weight and it is practically inoperative when the residue R is an alkyl group substituted by functional groups.

In addition, this procedure clearly is not suitable for preparing compounds of formula I in which R and/or R′ are fluorine atoms. It has now been found that it is possible to prepare compounds of general formula I in which R is an amino alkyl group and R′ is hydroxy with excellent yields and with a very high degree of purity when one reacts an aminoacid with phosphorous acid for the purpose of blocking the reactive amino group and then with phosphorus trichloride. The intermediate is then hydrolyzed and the product is isolated in an appropriate manner.

Instead of the mixture of phosphorous acid and phosphorus trichloride, it is possible to use only

phosphorus trichloride adding the stoichiometric amount of water in order to form the corresponding phosphorous acid.

Whenever it is possible, the aminoacid may be replaced by a precursor capable of forming the aminoacid by hydrolysis such as valerolactam or the corresponding polyamide in the case of 5-aminovaleric acid or the pyrrolidone in the case of 4-amino-butyric acid. The reaction is preferably carried out in the presence of an aprotic organic solvent such as an aliphatic or aromatic hydrocarbon or the corresponding chlorinated hydrocarbon, but may also be carried out in the absence of a solvent.

During the reaction, there is formed a pasty solid of composition not well defined from the which the desired aminobiphosphonic acid is obtained by hydrolysis with water or aqueous HCl.

The procedure described hereinabove is easily adapted to the industrial production of the acids of formula I. The preparation of the biphosphonic acids of general formula I in which R and R′ are both fluorine atoms may be carried out easily and with high yields by hydrolyzing the corresponding esters of general formula II:

$$F_2C[PO(OR'')_2] \qquad (II)$$

in which R″ is an alkyl residue which may be linear or branched containing between 1 and 4 carbon atoms. The esters of formula II are obtained by reacting the corresponding ester of the bromodifluoromethanphosphonic acid (which is obtained from dibromo-difluoromethane and a trialkylphosphite) with a dialkylphosphite of an alkali metal such as sodium according to the reaction scheme hereinbelow:

$$CF_2Br_2 + P(OR'')_3 \rightarrow Br—CF_2—PO(OR'')_2 \rightarrow NaPO(OR'')_2 \rightarrow II$$

The hydrolysis of the ester of formula II to the corresponding biphosphonic acid is carried out with water and mineral acid. The preferred compounds which are obtained according to the process of the present invention are:

5-amino-1-hydroxypentan-1,1-biphosphonic acid;
4-amino-1-hydroxybutan-1,1-biphosphonic acid;
difluoro-methanbiphosphonic acid;
and their sodium, aniline and lysine salts.

The following examples are described hereinbelow for the purpose of further illustration of the invention.

EXAMPLE 1

A mixture consisting of 117 grams (1.0 moles) of 5-amino-valeric acid, 123 grams (1.5 moles) of phosphorous acid and 500 cc of anhydrous chlorobenzene is prepared. The mixture is heated by means of a boiling water bath up to 100°C in a manner to solubilize the solid almost completely. Keeping the temperature at 100°C and under vigorous stirring, there are added slowly 206 grams (1.5 mole) of phosphorus trichloride. About 30 minutes after the end of the addition, the formation of a dense phase which has a tendency to increase and to harden with time begins. The mixture is kept for an additional three hours at 100°C and it is then allowed to cool under stirring. In this manner, the solid material breaks up into small pieces and may be filtered and washed with chlorobenzene. The hygroscopic solid so obtained is then dissolved in 500 cc of water and is heated for one hour under reflux. After cooling, the solution is treated with active carbon and then filtered. The crude acid precipitates by addition of an excess of warm methanol and after separation, the product is crystallized from one liter of water at 100°C.

The yield is 165 grams (63% of theory) of 5-amino-1-hydroxypentan-1,1-biphosphonic acid in the form of a white crystalline powder of melting point 235°C.

Elementary analysis
Found: C = 22.69; H = 5.71; N = 5.14; P = 23.70
calcd for $C_5H_{15}NO_7P_2$: C = 22.82; H = 5.75; N = 5.32; P = 23.54

Infrared Spectrum
Absorption bands at 3220, 1660 and 1510 cm^{-1}
Spectrum ^1H—N.M.R. (TMS as a standard)
δ = 1.8 ppm (6H); δ = 3.0 ppm (2H)

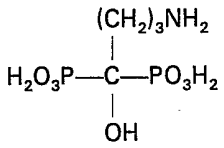
EXAMPLE 2

In a 150 liters glass reactor, there are introduced 9.4 kg of 5-aminovaleric acid, 9.9 kg of phosphorous acid and 40 liters of anhydrous chlorobenzene. The mixture is heated under stirring up to 90—100°C and 16.5 kg of phosphorus trichloride is added over a period of 30 minutes. The reaction mixture, after standing at 110°C for three hours, is cooled to 80°C and then 50 liters of water are added so as to dissolve all the solid material. The organic phase is allowed to cool and to separate from

the aqueous phase. After treatment of the aqueous phase with active carbon and filtration, excess methanol is added under stirring so as to precipitate the crude aminobiphosphonic acid. The mixture is filtered and the product is recrystallized from 60 liters of boiling water. The product is 12.4 kg of crystalline material of pure 5-amino-1-hydroxypentan-1,1-biphosphonic acid.

EXAMPLE 3

A mixture of 1 mole of 4-aminobutyric acid, 1.5 moles of phosphorous acid and 500 cc anhydrous chlorobenzene, is heated up to 100°C. At this temperature, phosphorus trichloride in the amount of 1.5 mole is added under strong stirring. The mixture is stirred at 100°C for $3\frac{1}{2}$ hours until the dense phase is completely formed and is then allowed to cool. The solid is filtered, washed with a small amount of chlorobenzene and dissolved in water. The solution is heated to the boiling point for one hour, it is then cooled and decolorized with active carbon. The material is filtered and the product is precipitated with excess of hot methanol. The crude material so obtained is heated under reflux for eight hours in 20% hydrochloric acid. The hydrochloric acid is removed by distillation and the residue is recrystallized from water. The product is 4-amino-1-hydroxybutan-1,1-bisphosphonic acid in the form of a white crystalline powder which has the structure hereinbelow as shown by the properties also reported hereinbelow:



Elementary analysis

	C%	H%	N%	P%
Found:	17.88	5.62	4.93	23.94
Calcd for ABDP:	19.28	5.26	5.64	24.86
Calcd for ABDP·H_2O:	17.98	5.66	5.24	23.19

Determination of the moisture content

The sample examined according to the Karl-Fischer method has a water content of about 3.9% by weight.

Potentiometric titration

The curve of potentiometric titration is obtained by addition of 0.1N NaOH to a solution of 203 mg of the sample dissolved in 75 cc of water. The profile of this curve is characterized by 2 clear end points at pH 4.4 and 9 corresponding to an addition of the reagent of 7.5 and 15.2 cc respectively. From the values reported, one calculates an equivalent weight of 270 for the first neutralization and 264 for the second neutralization and an average equivalent weight of 267. The molecular weight of ABDP·H_2O is 267.114.

Complexometric titration

The complexometric titration is carried out with thorium nitrate with 41.47 mg of the compound. It shows a color change after an addition of 5.4 cc of the reagent. From this value, it is possible to conclude that the substance being examined has an equivalent weight of 134, which is in agreement with the presence of two phosphonic groups in the molecole of the monohydrate.

Infrared absorption

The infrared spectrum observed on a tablet of KBr presents characteristic bands at:

3600—2500 cm^{-1}	complex band due the overlapping of the stretching of acidic and alcoholic OH groups, groups NH_3^+ and aliphatic CH
1650, 1605, 1500	bands due the deformation of the group NH_2 partially in the salt form
	due the presence of the phosphonic groups
1160	stretching of the P—O bond
1040	stretching of the C—O bond
960, 930	stretching of the P—O bond

600—400 skeleton bands which involve substantially the portion of the molecule which contains phosphorus atoms

Nuclear magnetic resonance of the Proton (^1H—NMR)

The ^1H—NMR spectrum calculated in D_2O/D_2SO_4 presents two enlarged signals at δ 2.6 ppm (CH_2-β and CH_2-γ due the NH_2 group) and 3.5 ppm (CH_2-α due the NH_2 group) for a relative intensity 2:1.

Nuclear magnetic resonance on carbon (^{13}C—NMR)

The spectrum ^{13}C—NMR determined in D_2O/D_2SO_4 presents signals at δ 20 ppm (CH_2-β due the NH_2 group), 28 ppm (CH_2-γ due the NH_2 group), 39 ppm (CH_2-α due the NH_2 group) and a central triplet at 72 ppm (C-δ due the NH_2 group, J_{C-P} 156 Hz).

Nuclear magnetic resonance on phosphorus (^{31}P—NMR)

The spectrum ^{31}P—NMR determined in the D_2O/D_2SO_4 presents a single signal at 9 ppm showing that two phosphorus atoms are chemically and magnetically equivalent.

EXAMPLE 4

Sodium diisopropylphosphite and diisopropyl bromodifluoromethanephosphonate are reacted according to conventional methods to produce the tetraisopropyl ester of difluoromethanebiphosphonic acid which is obtained as a colorless and odorless liquid of boiling point 117°C (0.2 torr).

Spectrum ^{19}F—N.M.R.: = 21.6 (triplet, CF_2, J_{F-P} = 86.67)
Spectrum ^{31}P—N.M.R.: = −16.1 (triplet, J_{F-P} = 14.6);
H_3PO_4 85% as the external standard.

The ester thus obtained is hydrolyzed to difluoromethanebiphosphonic acid which is obtained in crystalline form and is dried in a vacuum desiccator over P_2O_5. The substance is a very hygroscopic solid of melting point 90°C.

The curve of titration acid/base presents two clear end points at pH 3.9 and 10.1 which correspond respectively to the bisodium and tetrasodium salts and a single end point at pH 6.8 which corresponds to the trisodium salt. The molecular weight corresponding to the above titration values is 210.3 (theory 211.99).

EXAMPLE 5

To a suspension of 263 grams of 5-amino-1-hydroxy-pentan-1,1-biphosphonic acid in one liter of water is added under cooling a solution prepared from 40 grams of sodium hydroxide in 500 cc of water. There is obtained a clear solution which after decolorization with carbon, filtration and concentration is kept in the cold for a period of three days under gentle stirring. The crystalline solid thus obtained is filtered washed, with a small amount of cold water and then methanol. After drying at 110°C, there is obtained 199 grams of the monosodium salt of 5-amino-1-hydroxy-pentan-1,1-biphosphonic acid.

EXAMPLE 6

From difluoromethanebiphosphonic acid there is obtained, according to conventional methods, the trisodium difluoromethanebiphosphonate as a crystalline white powder soluble in water. The molecular weight determined by acid/base titration is 274.0 (theory 277.9). The 0.1 molar aqueous solution has pH = 6.8.

Elementary analysis
Found: C = 4.30; H = 0.52; P = 23.01; F = 12.90
Calcd for $CHF_2Na_3O_6P_2$: C = 4.32; H = 0.36; P = 22.29; F = 13.67.

EXAMPLE 7

From difluoromethanebiphosphonic acid there is obtained according to conventional methods the aniline difluoromethanebiphosphonate. The substance, after recrystallization from ethanol, melts at 163—165°C.

Elementary analysis
Found: C = 50.88; H = 6.02; N = 9.19; P = 9.90
Calcd for $C_{25}H_{32}F_2N_4O_6P_2 \cdot H_2O$ (tetraaniline salt as the monohydrate):
C = 50.80; H = 5.57; N = 9.11; P = 10.08.

UV Spectrum (in an aqueous solution)
Absorption maximum at 279 nm; $\varepsilon \cong 3241$.

EXAMPLE 8

From difluoromethanebiphosphonic acid there is obtained according to the method of Example 7, the lysine difluoromethanebiphosphonate. The dilysine salt, which precipitates from water, is obtained as a white amorphous powder, very soluble in water and hygroscopic. A 0.1 M solution has a pH = 4.0.

Elementary analysis
Found: C = 30.03; H = 6.42; N = 10.87; P = 13.01
Calcd for $C_{13}H_{32}F_2N_4O_{10}P_2$: C = 30.96; H = 6.39; N = 11.11; P = 12.28.

TOXICOLOGY STUDY

This study has been carried out with the following substances according to the present invention:
4-amino-1-hydroxybutan-1,1-biphosphonic acid (AHB_UBP);
5-amino-1-hydroxypentan-1,1-biphosphonic acid (AHP_EBP);
difluoromethanebiphosphonic acid as the sodium salt (F_2MBP).

By way of comparison the following substances have been used:
6-amino-1-hydroxyhexane-1,1-biphosphonic acid (AHE_XBP) prepared according to Italian Patent Application No. 19673 A/81 and dichloromethanebiphosphonic acid as the sodium salt (Cl_2MBP) (known).

Acute Toxicity

For this study Swiss mice, both male and female, have been utilized: during the experiment, the animals are fed according to the method with Altromin in the form of tablets. For the oral and intraperitoneal administration, there are used 5% gum arabic solutions, while saline solutions of pH 4 are used for the intravenous injections.

The preliminary values of DL_{50} are calculated according to a graphic method. Table 1 reports the values of the DL_{50} in Swiss mice in mg/kg.

TABLE 1

DL_{50} in Swiss Mice in mg/kg

	os	i.p.	i.v.
AHB_UBP	>2,000	—	85
AHP_EBP	1,500	75	85
AHE_XBP	>2,000	125	75
F_2MBP	>2,000	450	70
Cl_2MBP	>2,000	750	130

After the oral administration, also at the high dosage, no change in the behavior of the animal is observed, no death is noted and the only sympton is a certain softening of the stool. The autopsy of the animal killed shows a slight change in the kidneys which are of light and anemic color.

After the intravenous injection, the animals die immediately at the high dosage with convulsions and dyspnea. At the dosage lower than the lethal dose, the convulsions are less evident and continue for a period of two hours; the animals, after returning to normal, show a few cases of death after 2—4 days with dyspnea, hair erection and reduced motor activity. The autopsies show the kidneys with a pink or yellowish color with hemorragic spots, the female animals exhibit hypertrophic and hyperemic ovaries. The conclusion is that the novel biphosphonates exhibit acute toxicity and moderate chronic toxicity.

Inhibition of the formation of crystals

A model system is used to evaluate the ability of the phosphonates to inhibit the formation of crystals in inorganic solutions. Three solutions are prepared according to Fleisch.
1) 0.0107 M KH_2PO_4; 0.117 M KCl; 0.01 M barbituric acid
2) 0.0056 M $CaCl_2$; 0.138 M KCl; 0.01 M barbituric acid
3) 0.155 M KCl; 0.01 M barbituric acid.

The pH is brought to 7.4 by means of potassium hydroxide. The concentration of Ca^{++} was 6.7 mg%, a level similar to the calcium in blood which has been subjected to ultrafiltration and the concentration of the inorganic phosphate Pi give a product Ca^{++} x Pi = 80. The solution was analyzed for Ca^{++} and Pi and was distributed in Erlenmeyer flasks 12 cc each. The flasks were divided in groups as follows:

a) Control	
b) $AH_{EX}BP$	0.05 μM
c) $AH_{EX}BP$	0.25 μM
d) $AH_{EX}BP$	0.5 μM
e) $AH_{EX}BP$	2.5 μM
f) $AH_{EX}BP$	5.0 μM
g) Cl_2MBP	0.5 μM
h) Cl_2MBP	2.5 μM
i) Cl_2MBP	5.0 μM
l) F_2MBP	0.5 μM
m) F_2MBP	2.5 μM
n) F_2MBP	5.0 μM
o) AHP_EBP	0.05 μM
p) AHP_EBP	0.25 μM
q) AHP_EBP	0.5 μM
r) AHP_EBP	2.5 μM
s) AHP_EBP	5.0 μM
t) AHB_UBP	0.5 μM
u) AHB_UBP	2.5 μM
v) AHB_UBP	5.0 μM

and then they are incubated under stirring at 37°C for two days.

At the end of incubation, the solutions are passed through "millipore filters" for the purpose of retaining the crystals which are formed during the incubation; the filtrate then is analyzed for Ca^{++} and Pi. The results are also reported in Table 2 as the product of Ca^{++} x Pi in the solution at the end of the experiment.

The data show that the biphosphonates according to the present invention induce a significant inhibitory activity on the formation and growth of crystals of apatite according to a pattern which is dependent on the dose.

TABLE 2

Substance	Conc. μM	Values of the product Ca^{++} x Pi in solution: Prior to Incubation	After Incubation
Control	0.0	114.7	29
Cl_2MBP	0.5	"	44.0
Cl_2MBP	2.5	"	60.4
Cl_2MBP	5.0	"	73.6
F_2MBP	0.5	"	44.6
F_2MBP	2.5	"	58.5
F_2MBP	5.0	"	72.0
AHE_XBP	0.05	"	30.0
AHE_XBP	0.25	"	37.5
AHE_XBP	0.5	"	59.6
AHE_XBP	2.5	"	92.6
AHE_XBP	5.0	"	95.3
AHP_EBP	0.05	"	36.7
AHP_EBP	0.25	"	37.1
AHP_EBP	0.5	"	68.6
AHP_EBP	2.5	"	94.0
AHP_EBP	5.0	"	97.5
AHB_UBP	0.5	"	53.2
AHB_UBP	2.5	"	88.6
AHB_UBP	5.0	"	93.5

Pharmacological tests

The purpose of this study is to investigate the effect of a series of novel biphosphonates on a culture of skull cells and on the bone reabsorption and the mineralization in vivo.

Methods used

1. Experiments on skull cells

Cellular culture: the cells are cultured according to the method described by Fast et al, (Biochem. J. *172*, 97—107 (1978)). By way of summary, the skulls removed from Wistar rats, one day old, are digested with collagenase. The cells set free are placed on a plate with concentration of 200,000 cells per cc of medium in disks "clusters" suitable for culture, the plates having 24 wells of 1.6 cm in diameter containing 0.5 cc of medium. The cells are cultivated in the essential minimum medium containing 10% of foetal calf serum in an atmosphere of 5% CO_2 at 37° up to the eighth day. The biphosphonates are added on the first day up to the end of the experiment. The medium is changed on the first, fourth and seventh day.

Cellular count

The cells are counted with a Coulter counter after they have been set free from the disks by digestion with a mixture of collagenase and trypsin.

Determination of lactate

On the seventh day, the medium is changed and the cells are incubated for 16 hours. The lactate produced during this period is measured in an extract in $HClO_4$ of the medium using lactatodehydrogenase.

2. Experiments on the bone reabsorption and in vivo calcification

Groups of five Wistar rats of weight 180—200 grams are treated for a period of seven days with 0.1, 1.0 and 10 mg of P/kg of the following biphosphonic acids:
difluoromethanebiphosphonic acid (F_2MBP) (in the form of Na salt);
4-amino-1-hydroxybutanbiphosphonic acid (AHB_UBP);
5-amino-1-hydroxypentanbiphosphonic acid (AHP_EBP);
6-amino-1-hydroxyhexanebiphosphonic acid (AHE_XBP);
dichloromethanebiphosphonic acid (Cl_2MBP) (in the form of the sodium salt) (1 mg of P/kg).

The animals kept as a control were administered the solvent with NaCl. All the treatments were carried out by the subcutaneous route. The compounds were dissolved in NaCl for the two lower concentrations and in water for the higher concentration and were administered in a volume of 0.2 ml/100 g. The animals were fed with Altromine 1314 containing 1.1 g/100 g P and 250 IU/100 g of vitamin D_3. On the eighth day, the animals were killed and the tibia was removed and fixed in 50% ethanol. The tibiae were then dehydrated in increasing concentration of ethanol and allowed to soak in methylmethacrylate after the addition of Plastoid N. Frontal sections were removed and cut to a thickness of 70—80 μm and then the sections were submitted to microradiography. This procedure permitted the evaluation of the density of the mineral in the trabecular metaphysis according to the method of Shenk et al., Calc. Tiss. Res. *11*, 196—214, 1973).

Results

1. Experiments with skull cells

As shown in Table 3, Cl_2MBP causes a decrease in the number of cells. On the other hand, F_2MBP has no effect or a very small effect in this respect. The aminoderivatives show a difference because the compounds with an odd number of carbon atoms decrease the cellular number to a much greater extent than the compounds with an even number of carbon atoms.

TABLE 3

Effect on the cellular number ± S.E.M. (n)

Composition	% of control concentration (μM)		
	2.5	25	250
Cl_2MBP	103.0 ± 0.7 (4)	86.4 ± 2.1 (12)***	54.5 ± 1.9 (12)***
F_2MBP	88.1 ± 1.4 (12)***	92.4 ± 1.9 (12)***	99.3 ± 2.0 (16)
AHB_UBP	100.5 ± 1.6 (8)	101.0 ± 1.5 (7)	74.2 ± 4.7 (15)***
AHP_EBP	102.7 ± 2.8 (8)	42.6 ± 5.1 (16)***	dead cells
AHE_XBP	93.3 ± 3.0 (8)*	95.2 ± 2.2 (8)	87.0 ± 3.4 (20)*

Number of cells of control: 0.5548 10^6 disk ± 0.05 (55) ($\bar{x}$ ± SEM (n))

By reference to the production of the lactate Cl_2MBP diminishes it as is well known. On the other hand, F_2MBP has no effect. The amino derivatives exhibit increase in the production of the lactate, a fact which is more pronounced with the compounds with an odd number of carbon atoms. The data are reported in Table 4.

TABLE 4

Effect on the production of lactate ± S.E.M. (n)

	% of control concentration (μM)		
Compound	2.5	25	250
Cl_2MBP	87.5 ± 4.0 (4)*	67.1 ± 2.8 (12)***	16.9 ± 2.2 (12)***
F_2MBP	112.1 ± 4.4 (12)*	106.2 ± 3.9 (12)	99.7 ± 4.7 (16)
AHB_UBP	88.0 ± 1.5 (8)*	79.6 ± 4.1 (7)***	179.8 ± 18.6 (15)**
AHP_EBP	93.4 ± 3.0 (8)	354.1 ± 27.7 (16)***	dead cells
AHE_XBP	109.4 ± 6.0 (8)	108.3 ± 4.5 (8)	164.7 ± 7.7 (20)***

+ In one experiment, the number of cells was 51.2% of the control, in three experiments, it was 1—3%. These concentrations represent the limit at which the cells die.

Production of lactate: 3.83 μMol/10^6 cells ± 0.10 (55) ($\bar{x}$ ± SEM (n))

Experiments on the bone reabsorption and calcification in vivo

One animal per group has been evaluated. The data are reported in Table 5.

TABLE 5

Effect on the bone reabsorption and mineralization of bones

Compound	Dose (mg)	Reabsorption	Mineralization
F_2MBP	10	–	–
	1	–	–
	0.1	–	–
AHB_UBP	10	+++	–/+
	1	++/+++	–
	0.1	+	
AHP_EBP	10	Experiment interrupted due to acute toxicity	
	1	+++	–
	0.1	+++	–
AHE_XBP	10	*	+++
	1	++	–
	0.1	–	–
Cl_2MBP	1	+/++	–

– = no inhibition of the reabsorption or mineralization
Between + and +++ = increase in the inhibition of reabsorption or mineralization
* = effect not established due to inhibition of mineralization

It appears that AHP_EBP is the most active in inhibiting the bone reabsorption. However, there is observed a toxicity at the higher dosage. The substances AHP_UBP and AHE_XBP are also active on the reabsorption with a result slightly superior to Cl_2MBP. A significant difference is with respect to the mineralization because AHE_XBP induces strong inhibition of mineralization in the dose of 10 mg of P/kg while AHB_UBP has no effect or only a slight effect or only an effect to a very small extent.

These results show that the amino compounds with an odd number of carbon atoms are somewhat toxic but are much more active in inhibiting the bone reabsorption. The compounds with an even number of carbon atoms have an activity slightly superior to Cl_2MBP. Another significant fact is that AHB_UBP does not induce or induces only to a very small extent the inhibition of mineralization at high dosage while AHE_XBP exhibits high inhibition. Consequently, AHB_UBP appears to be more suitable for use in diseases with an increased reabsorption of bone in humans. Finally, it is interesting to note that F_2MBP has no effect on the bone reabsorption or on the bone mineralization and in view of the fact that it inhibits the growth *in vitro* of the crystals of apatite, it may be used successfully in conditions of urolithiasis.

In fact, for a long time, a biphosphonate capable of inhibiting the growth of the crystals without affecting the bone has been the subject of research. It is concluded, therefore, that the two substances AHB_UBP and AHP_EBP are destined to become medicaments capable of inhibiting the bone reabsorption and that F_2MBP is useful for the treatment of urolithiasis.

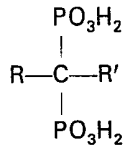
The pharmaceutical compositions according to the present invention may be prepared for use in the form of capsules or tablets or in solution for oral administration or for systemic use. The compositions are advantageously prepared together with inert carriers such as sugars (saccharose, glucose, lactose), starch and derivatives, cellulose, and derivatives, gums, fatty acids and their salts, polyalcohols, talc, aromatic esters.

The pharmaceutical compositions can be administered by the oral route at doses from 25 to 3200

mg/die or by the parenteral route at doses from 15 to 300 mg/die. Treatment is carried out for 7 days or for 3 months' courses, repeated according to needs.

CLAIMS

1. A pharmaceutical composition which contains as the active ingredient at least one biphosphonic acid of general formula I:



(I)

wherein R is a fluorine atom or a linear or branched alkyl containing from 1 to 5 carbon atoms, said alkyl being unsubstituted or substituted by at least one amino group, and/or fluorine atom and R' is hydroxy or fluorine, or salt thereof with an alkali metal or an organic base or a basic amino acid.

2. A pharmaceutical composition according to claim 1 in a form suitable for oral administration.

3. A pharmaceutical composition according to claim 1 in a form suitable for systemic administration.

4. A process for the preparation of a biphosphonic acid of general formula (I) according to claim 1 wherein R is an aminoalkyl residue and R' is hydroxy which comprises reacting an amino acid or precursor thereof with phosphorous acid and phosphorus trichloride in an inert solvent, hydrolyzing the reaction product and isolating said biphosphonic acid from the reaction mixture.

5. The process according to claim 4 wherein said amino acid and phosphorous acid and said phosphorus trichloride are reacted in the ratio of 1:1,5:1,5.

6. The compound 5-amino-1-hydroxypentan-1,1-biphosphonic acid.

7. The compound 4-amino-1-hydroxybutan-1,1-biphosphonic acid.

8. The compound difluoromethanbiphosphonic acid.

9. A pharmaceutical composition according to claim 1 substantially as described herein and exemplified.

10. A process for the preparation of a biphosphonic acid of formula (I) substantially as described herein and exemplified.

11. A biphosphonic acid or a salt thereof when prepared by the process of claims 4, 5 or 10.

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