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Anion-exchange separation and determination of bisphosphonates and related analytes by post-column indirect fluorescence detection

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Abstract

Bisphosphonic acids and their salts can be detected after their liquid chromatographic separation by post-column indirect fluorescence detection (IFD). After separation the analyte is combined with the highly fluorescent Al^{3+}–morin (2′,3,4′,5,7-pentahydroxyflavone) solution and fluorescence decreases because of the formation of the nonfluorescent Al^{3+}–bisphosphonate complex. The decrease in fluorescence is proportional to the amount of bisphosphonate present. Separation of the multivalent anionic bisphosphonate analytes from other anions and sample matrix is achieved on a strong base anion-exchange column with a strong, basic eluent. The post-column reaction variables, which influence IFD, are identified and optimized for the detection of the bisphosphonates after separation on the anion exchanger. The method is selective, since only a few anions will undergo a reaction with the Al^{3+}–morin solution, and sensitive, detection limit for difluoromethylene bisphosphonate, F_2MDP, is 4 ng for $S/N=3$. The separation–IFD method can be applied to the determination of bisphosphonates, such as F_2MDP, ethane-1-hydroxy-1,1-bisphosphonic acid, dichloromethylene bisphosphonic acid, 4-amino-1-hydroxybutane-1,1-bisphosphonic acid, in biological samples. The separation–IFD method is also applicable to the detection of inositol phosphate anions.

Keywords: Derivatization, LC; Detection, LC; Indirect fluorescence detection; Fluorescence detection; Bisphosphonates; Phosphonates; Inositol phosphate

1. Introduction

Bisphosphonic acids and their bisphosphonate salts are analogs of pyrophosphate where the P–O–P linkage of the latter is replaced by the P–C–P linkage. Initial interest in the bisphosphonates focused on the synthesis of ethane-1-hydroxy-1,1-bisphosphonic acid (EHDP) and its potential application as a detergent additive. Broad physiological effects of EHDP were discovered [1] and subsequently several other bisphosphonic acids and their salts have been synthesized and their physiological effects are under evaluation. The bisphosphonates bind strongly to Ca^{2+} [2,3] and this inhibits the breakdown of bone by cell osteoclasts. Initially, it was thought that the bisphosphonates like EHDP are incorporated into the system via replacement of naturally occurring pyrophosphate [1], however, as other bisphosphonate derivatives, particularly those containing a side chain N-group, were synthesized and evaluated other physiological processes were suggested to occur [4–8]. At present several bisphosphonates have been shown to be effective in the treatment of osteoporosis, Paget's disease, other Ca^{2+} disorders, acquired immunodeficiency syndrome (AIDS), and certain cancers.

Bisphosphonic acids, such as EHDP, dichloromethylene bisphosphonic acid (Cl_2MDP), di-

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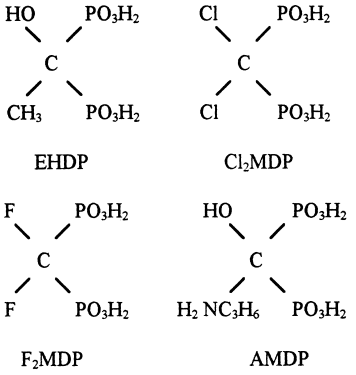
fluoromethylene bisphosphonic acid (F_2MDP) and 4-amino-1-hydroxybutane-1,1-bisphosphonic acid (AMDP), which are usually employed as their salts, are difficult to determine in biological samples at the concentration levels that are typically encountered in studies of their physiological effects.



The bisphosphonates do not absorb or fluoresce and detection strategies based on these properties are therefore not applicable. Furthermore, when faced with the determination of the bisphosphonates in biological samples the biological sample matrix often causes a significant interference.

Analytical methods that were initially used for the determination of the bisphosphonates include the titration of the bisphosphonic acids as acids [1–3], titration with a Th^{4+}–diaminocyclohexanetetraacetate complex titrant and with a spectrophotometric end point detection [9], and spectrophotometrically after conversion of the bisphosphonate to phosphate followed by the formation of phosphomolybdate [10]. However, these methods are not sensitive enough for the determination of the bisphosphonates at trace levels.

Chromatographic methods have been shown to be sensitive for the determination of bisphosphonates and can be applied to their determination in biological samples and pharmaceutical products. Gas chromatography (GC) has been used for the determination of 3-amino-1-hydroxypropane-1,1-bisphosphonate and the 6-amino-1-hydroxyhexane-1,1-bisphosphonate after derivatization to form the volatile *N*-iso-*tert*.-butoxycarbonyl (*N*-iso-Boc) methyl ester derivative. Detection limit was 100 pg for a S/N of 3 [11]. EHDP was also determined in tablets by GC after conversion to the trimethylsilyl derivative [12]. Procedures for the liquid chromatographic determination of bisphosphonates with direct detection or derivatization to enhance detection have been reported. Bisphosphonates were derivatized pre-column with fluorescamine, separated on a C_{18} column, and the derivatives detected by fluorescence [13,14] or detected by fluorescence after post-column reaction with *o*-phthaladehyde [15]. The reagent 2,3-naphthalene dicarboxyaldehyde-cyanide was used to derivatize AMDP in urine samples and the derivative could be detected after separation on a C_{18} column by fluorescence at a sensitivity of 5 ng/ml [16]. Bisphosphonates after separation were converted post-column to orthophosphate which was then converted to phosphorus vanadomolybdeum acid and detected at 410 nm [17] or to the phosphomolybdate and detected at 820 nm [18]. The absorbance change that occurs after a post-column reaction of the bisphosphonate analyte with a $Fe(ClO_4)_3$ solution could be used for detection; detection limit, however, is only 500 ng [19].

Direct detection methods have been reported for the liquid chromatographic separation and determination of bisphosphonates. Cl_2MDP was determined in serum and urine using flame photometric detection [20]. Methylene bisphosphonate was separated on a C_{18} column with a mobile phase containing trioctyl amine as a pairing reagent using refractive index for detection [21]. Separation on an anion-exchange column and refractive index detection was also used in the liquid chromatographic determination of 3-amino-1-hydroxypropane-1,1-bisphosphonate in injectables, capsules and tablets [22]. Detection limit was about 20 pg. Conductivity detection [23], inductively coupled plasma detection [24], and indirect UV absorbance detection [25] was used for the ion chromatographic separation of bisphosphonates in dosage formulations on an anion-exchange column. Liquid chromatography–mass spectrometry (LC–MS), employing an anion-exchange column to separate AMDP, was used to characterize AMDP and establish its dominate fragmentation pathway [26].

Morin (2′,3,4′,5,7-pentahydroxyflavone), which is fluorescent, undergoes a reaction with Al^{3+} and other metal ions to form a more intense fluorescent complex and is the basis for their determination [27]. When either fluoride or phosphate anions are introduced into a Al^{3+}–morin solution, morin is released

due to the formation of the stronger Al^{3+}–fluoride or –phosphate complex and fluorescence decreases. This decrease in fluorescence can be correlated to fluoride [28] or phosphate [27] concentration in the sample and this methodology is the basis for a quantitative determination of the two anions.

The Al^{3+}–morin complex was evaluated as a post-column reagent for the indirect fluorescence detection (IFD) of phosphate and certain organophosphonates [29] and fluoride ion [30] following a liquid chromatographic separation. In this separation–detection strategy the analyte anions are separated from other anions and sample matrix components on a strong base anion-exchange column and subsequently detected post-column by IFD with the Al^{3+}–morin solution. A decrease in fluorescence that is measured is proportional to the amount of the analyte anion that is in the sample and separated by the column. The Al^{3+}–morin IFD separation–detection procedure was also shown to be applicable to the separation and determination of *N*-(phosphonomethyl)glycine (glyphosate) and its principle metabolite/decomposition product 1-amino-1,1-diphosphonic acid [31] and for fluorophosphate anion in tooth paste [30].

2. Experimental

2.1. Reagents

The disodium salt of F_2MDP was synthesized [32] while disodium Cl_2MDP and disodium EHDP were obtained from the College of Pharmacy, University of Iowa. The disodium salt of AMDP was obtained from Merck, Sharp and Dohme and D,L-myo-inositol-1-monophosphate, cyclohexylammonium salt, was purchased from Sigma. Morin hydrate (2′,3,4′,5,7-pentahydroxyflavone) was supplied by Aldrich. Embryonic bone samples, culture media and related reagents were prepared in the College of Dentistry, University of Iowa [33]. Acids, bases, $Al(NO_3)_3$ and 95% ethanol were purchased as analytical grade when possible. LC grade water was prepared by passing laboratory-distilled water through a Millipore Milli-Q Plus water system.

2.2. Instrumentation

A Spectra Physics Model 8800 gradient pump was used to deliver the mobile phase. The injector was a Model 7125 Rheodyne injector equipped with different sample loops. Fluorescence detection was with a Kratos 9000-9501 fluorescence detector equipped with a Kratos blue band excitation filter (FSA 404) (400 to 470 nm) and a Kratos filter with 50% transmission at 480 nm as the emission filter. The column was a polystyrene–divinylbenzene quaternary ammonium type strong base anion exchanger obtained from Hamilton (Hamilton PRP-X100, 150 mm×4.1 mm I.D., 10 μm, 0.19 mequiv./g exchange capacity). The column efficiency for the separation of a F^-, Cl^-, NO_3^- test mixture was greater than 10 000 plates/m for an aqueous 2 m*M* sodium benzoate mobile phase at pH 6.5 and a 2.0 ml/min flow-rate.

A Varian Model 2010 pump was used to deliver the post-column Al^{3+}–morin reagent through a pulse damper consisting of an empty 250 mm×8.0 mm I.D. column, a 150 mm×4.6 mm I.D. column packed with 80 mesh glass beads, and a 2 m×0.508 mm I.D. coil of stainless steel tubing into a Lee Visco mixing tee (No. 344790SN152) where the post-column reagent was mixed with the column effluent. The resulting mixture was passed through a knitted reaction coil of 0.762 mm I.D. polyether ether ketone (PEEK) tubing (Upchurch Chromatography) providing a volume of usually about 650 to 700 μl and then into the fluorescence detector. The coil temperature was usually maintained at 70°C except where noted. Data were recorded and/or collected by integrator. Peak areas are reported in relative integrator units.

2.3. Procedures

Standard solutions of the bisphosphonate sodium salts were prepared by dissolving known weights of the samples in a known volume of distilled water. Known aliquots of these stock solutions were diluted and used to prepare solutions of analyte test samples and calibration curves. Sample injections were 5 to 25 μl with a Hamilton 702 syringe or with a fixed-volume sample loop.

Mobile phases were aqueous solutions containing known amounts of $NaNO_3$ and NaOH. The post-

column Al^{3+}–morin solution was made by combining known aliquots taken from stock solutions of morin, $Al(NO_3)_3$, and HOAc–NaOAc with 95% ethanol and LC water to yield a 2 μ*M* $Al(NO_3)_3$, 12 μ*M* morin, 37 m*M* HOAc–NaOH buffer, ethanol–water (80:20) solution. The post-column reagent solution was stored overnight prior to use to ensure formation of the Al^{3+}–morin complex. The solution was found to be stable for at least two months when it was stored in a closed container. Delivery rate for the post-column Al^{3+}–morin reagent solution was usually 1.0 ml/min. The excitation and fluorescence wavelengths for the Al^{3+}–morin reagent solution at pH 4.5 were 420 and 505 nm, respectively.

3. Results and discussion

3.1. Separation–detection

The bisphosphonic acids are modestly strong tetraprotic acids which are multivalent anions at high pH [3]. Thus, they are highly retained on an anion-exchange column from a basic mobile phase and a strong eluent is required for their elution from the anion-exchange column. Because of this, the bisphosphonates are readily separated from weakly retained inorganic and organic anions. In the procedure described here the bisphosphonate analytes are retained while other anions and matrix interferences are eluted from the strong base anion-exchange column. Subsequently, the bisphosphonate analytes are then eluted from the anion-exchange column. The effluent from the column is combined post-column with a fluorescent Al^{3+}–morin solution and the resulting mixture is passed through a fluorescence detector set at a wavelength where the Al^{3+}–morin complex fluoresces. When the bisphosphonate analyte emerges from the column and is combined with the post-column Al^{3+}–morin reagent solution a new equilibrium position is established in the analyte band according to Reaction 1.

$$Al^{3+}\text{–morin} + \text{bisphosphonate analyte} \rightleftharpoons Al^{3+}\text{–bisphosphonate analyte} + \text{morin} \quad (1)$$

The bisphosphonate analyte replaces morin in the complex because the bisphosphonate forms a stronger complex with Al^{3+}. This causes the fluorescence in the analyte band to decrease since the Al^{3+}–bisphosphonate complex is nonfluorescent. Thus, the fluorescence decrease on the continuous fluorescent background produces a negative chromatographic peak whose peak height and peak area are proportional to the bisphosphonate analyte concentration. This IFD strategy combined with the anion-exchange separation is both sensitive, due to detection of a fluorescent change, and selective because of two factors. First, the bisphosphonate analytes are retained on the anion-exchange column at a strong eluent condition where most other anions and sample matrix components are readily eluted. Second, few analytes will replace morin in the Al^{3+}–morin complex.

The typical mobile phase depending on the bisphosphonate analyte was an aqueous 40 m*M* $NaNO_3$, 4.1 m*M* NaOH solution. The $NaNO_3$ concentration in the mobile phase can be reduced to obtain a weaker eluent or increased for a stronger eluent. A modest change in the $NaNO_3$ concentration in the mobile phase has no effect on IFD.

3.2. Post-column variables

Typically, a post-column derivatization direct detection strategy involves a post-column reaction where the analyte is converted into a detector active species. Thus, detector response increases as analyte concentration increases and this is the basis for a quantitative calibration. In the post-column IFD strategy used in this report the fluorescent background of the post-column solution decreases as the analyte concentration increases and the fluorescent decrease is correlated to analyte concentration. Post-column indirect detection like a post-column derivatization direct detection requires careful control of the conditions for the post-column reaction, which is the basis for the analyte detection. In addition a detector capable of offsetting the background signal that is present is necessary in order to obtain a favorable detection limit.

The post-column variables are of two types. Post-column solution pH, solvent composition, Al^{3+}:morin ratio, and Al^{3+}–morin complex concentration are variables that strongly influence background fluorescence and the equilibrium position in

Reaction 1. Post-column reaction temperature, reagent flow-rate, reaction coil volume and buffer concentration are variables that have a major influence on the rate of reaction described by Reaction 1. All of these variables were experimentally optimized to obtain the most sensitive IFD of the bisphosphonates following their separation on an anion-exchange column where optimum mobile conditions were used for the separation. This was done by systematically varying each variable while the others were held constant at their optimum conditions.

The background fluorescence intensity for the Al^{3+}–morin solution is pH dependent as is the competitive reaction between morin and the bisphosphonate analyte towards Al^{3+} (see Reaction 1). The largest peak area is obtained for the bisphosphonate analyte when the post-column solution pH is between pH 4.1 to 4.5. Because the mobile phase used for the separation of the bisphosphonate analytes on the anion-exchange column is basic, the post-column reagent solution must be a buffer of sufficient buffer capacity to lower the column effluent pH into this optimum pH range. A 37 m*M* acetate buffer was shown to be optimum for the post-column reagent solution to yield a post-column pH of 4.3 for the elution and post-column mixing conditions used. Excess acetate buffer should be avoided since bisphosphonate analyte peak area will decrease with increasing acetate concentration in the post-column mixture.

Morin has low water solubility and ethanol is required in the post-column reagent solution to maintain morin solubility in the reagent solution and also when the reagent solution is mixed with the column effluent. Ethanol also influences complex formation constants as will as the rate of complex formation for the Al^{3+}–morin and Al^{3+}–bisphosphonate analyte complex. For the post-column reagent solution and column effluent mixing ratio used an optimum peak area for the bisphosphonate analytes is obtained when the post-column reagent is ethanol–water (4:1). This corresponds to about 40% ethanol in the post-column reagent solution and column effluent mixture when both the mobile phase and the post-column Al^{3+}–morin reagent solution are 1.0 ml/min.

The peak area for a bisphosphonate analyte increases as the morin concentration increases in the Al^{3+}–morin post-column reagent solution up to the ratio of about 1:3 Al^{3+}:morin then increases gradually for higher amounts of morin. This change is illustrated in Fig. 1 where F_2MDP peak area is plotted versus the morin to Al^{3+} ratio. Increasing the morin above the 1:5 Al^{3+}:morin ratio offers little advantage in bisphosphonate analyte detection and actually causes the detection limit to be poorer due to an increase in the background fluorescence noise since the morin itself fluoresces at the wavelength used. Excess morin concentration in the mobile phase will also exceed the offset capabilities of the fluorescence detector. In the experiments described here the post-column reagent solution was typically 1:4 to 1:5 Al^{3+}–morin.

Peak area for the bisphosphonate analytes is influenced by the concentration of the Al^{3+}–morin complex in the post-column reagent solution. Increasing the Al^{3+} concentration while maintaining the 1:5 Al^{3+}:morin ratio causes a sharp increase in bisphosphonate analyte peak area up to about 4 μ*M* Al^{3+} in the post-column reagent solution and then gradually increases with Al^{3+} concentration. However, as the Al^{3+}–morin concentration is increased,

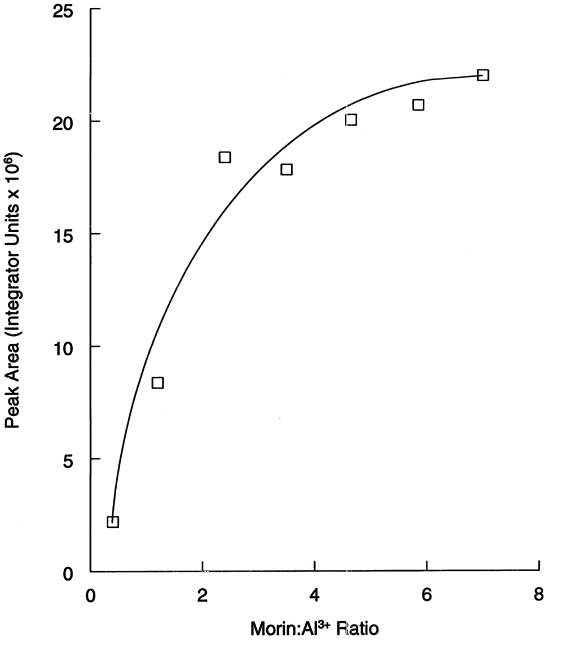


Fig. 1. Effect of the ratio of morin to Al^{3+} on F_2MDP peak area.

background fluorescence also rises and the problem of electronically offsetting the background fluorescence with the detector increases. Thus, the background fluorescence noise increases which causes a less favorable detection limit. An optimum IFD condition is therefore obtained, which was used in these experiments, when Al^{3+} concentration is about 2 μ*M* and the Al^{3+}:morin ratio is about 1:5.

Post-column reaction coil temperature has two opposing effects on detection. The fluorescent intensity for the Al–morin complex is temperature dependent and its formation constant decreases as temperature increases [34]. Thus, fluorescent intensity of the complex decreases as the temperature is increased. This results in a reduction in the difference between the Al–morin complex solution fluorescence and the fluorescence of the solution after the bisphosphonate analyte replaces the morin in the complex according to Reaction 1. Since the difference in the two fluorescence quantities is the origin of the bisphosphonate analyte detection, a decrease in the difference has an adverse effect on the detection limit. Thus, this effect requires a lower temperature. However, the rate of reaction between the Al–morin and the bisphosphonate analyte shown in Reaction 1 is also temperature dependent and increases as temperature increases. The further the reaction in Reaction 1 proceeds to completion, the greater the difference between the background fluorescence due to the Al–morin and the reduced fluorescence exhibited by the analyte band due to the formation of the Al–bisphosphonate analyte complex, the more favorable the detection limit becomes.

As post-column temperature increases bisphosphonate analyte peak area increases and reaches a broad maximum at about 70°C due to the two opposing effects. All measurements reported here were made with the post-column reaction taking place in the reaction coil which was placed in an oven maintained usually at 70°C. Also, the optimum bisphosphonate analyte peak area for the post-column reaction was obtained when the column effluent at 1.0 ml/min was combined with the post-column reagent solution which was also at 1.0 ml/min.

The rate of the replacement of the morin by the bisphosphonate analyte in the Al–morin complex described in Reaction 1 is time dependent. For the optimum flow-rate of 1.0 ml/min for both the mobile phase flow-rate and the post-column Al^{3+}–morin reagent solution flow-rate, an optimum coil tubing I.D., and a reaction temperature of 70°C, an increase in the volume of the reaction coil produces a longer reaction time. Therefore, the difference between the background fluorescence and the fluorescence in the bisphosphonate analyte band is greater and the detection limit is more favorable. However, if the volume of the reaction coil is to large, band broadening occurs which has an adverse effect on detection limit. The effects of post-column reaction volume and temperature is analyte dependent and should be optimized for each specific analyte [28–30]. The variables that are reported here are optimized for the IFD of the bisphosphonate analytes after their retention, separation and elution from an anion-exchange column with the 40 m*M* $NaNO_3$, 4.1 m*M* NaOH mobile phase.

Reaction volume and subsequently reaction time can be changed by either changing the reaction coil length or I.D. For a 0.762 mm I.D. PEEK tubing reaction coil at 70°C bisphosphonate analyte peak area increased as the length (and volume) of the reaction coil is increased. This is illustrated in Fig. 2 where bisphosphonate analyte peak area is plotted

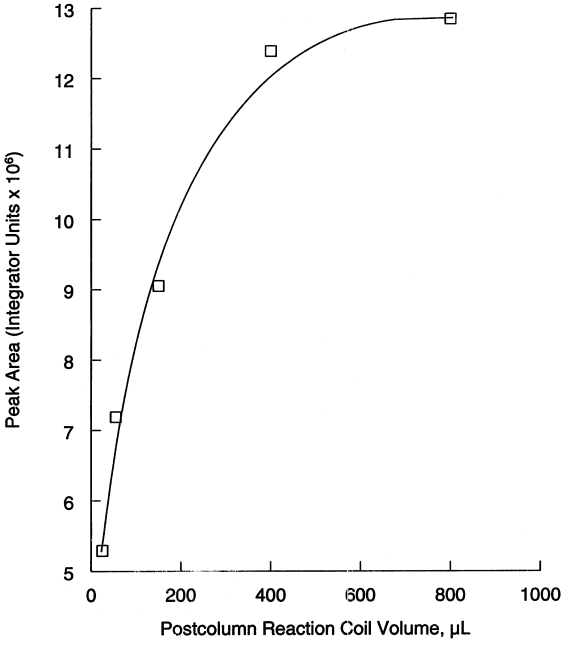


Fig. 2. Effect of the reaction coil volume on F_2MDP peak area.

versus reaction coil volume. The optimum reaction coil volume is about 600 μl, which for the 0.762 mm I.D. coil tubing, corresponds to a tube length of about 1.3 m. If a wider I.D. coil tube is used to reduce coil length, background fluorescence noise, which was shown to be due to the pumping of the post-column reagent solution, was significantly increased. Also, when stainless steel tubing was used for the reaction coil, bisphosphonate analyte chromatographic peak distortion occurred over time and increased with continued use of the stainless steel coil even though the analytical performance of the anion-exchange column was shown to be constant. A flow injection procedure was used to experimentally demonstrate that analytes were being retarded by the stainless steel coil. This problem was eliminated by employing PEEK tubing for the reaction coil and the post-column connecting tubing.

3.3. Bisphosphonate analysis

Relatively strong eluting conditions are used for the retention and subsequent elution of the bisphosphonate analytes from the anion-exchange column. Thus, most inorganic anions are quickly eluted from the column, if present in the sample, and do not interfere in the detection of the bisphosphonate analytes. Even phosphate anion which has some retention for the mobile phase conditions used will not interfere [29] unless it is in large excess; in this case it will overlap with some of the less retained bisphosphonate analyte peaks. Furthermore, IFD is a selective detection strategy and only those analytes which compete with morin in the reaction with Al^{3+} (see Reaction 1) will be detected. Thus, the selectivity offered by IFD combined with the anion-exchange separation overcomes the potential interferences that would be encountered with biological samples. The analytical anion-exchange column typically delivered over 10 000 plates/m for both the test mixture and the bisphosphonate analytes and the analyte peak broadening that occurred is largely due to the post-column reaction conditions.

Fig. 3A illustrates the separation of pyrophosphate and F_2MDP on the PRP-X100 anion-exchange column where both analytes are detected by IFD. Biological samples, particularly bone samples, are likely to have pyrophosphate in the sample which could possibly be an interference. Because a strong eluent condition of 40 m*M* $NaNO_3$, 4.1 m*M* NaOH is used pyrophosphate elutes early and consequently does not interfere in the determination of F_2MDP. Fig. 3B illustrates the chromatogram obtained for the elution of a Cl_2MDP standard on the PRP-X100 anion exchanger using an aqueous 40 m*M* $NaNO_3$, 4.1 m*M* NaOH mobile phase and IFD. A calibration curve for the Cl_2MDP standard was prepared and determined to be linear up to 455 ng of injected Cl_2MDP (upper limit of linearity was not established). The curve was defined by the equation, Cl_2MDP peak area = 6490.5 ng Cl_2MDP-136 870 with an r value of 0.9991. The detection limit for $S/N = 3$ was 6 ng. Cl_2MDP was subsequently determined in embryonic rat bones that had been incubated in cell growth media containing Cl_2MDP and found to be 0.95 μg of Cl_2MDP per mg of embryonic rat bone. The bone samples were hydro-

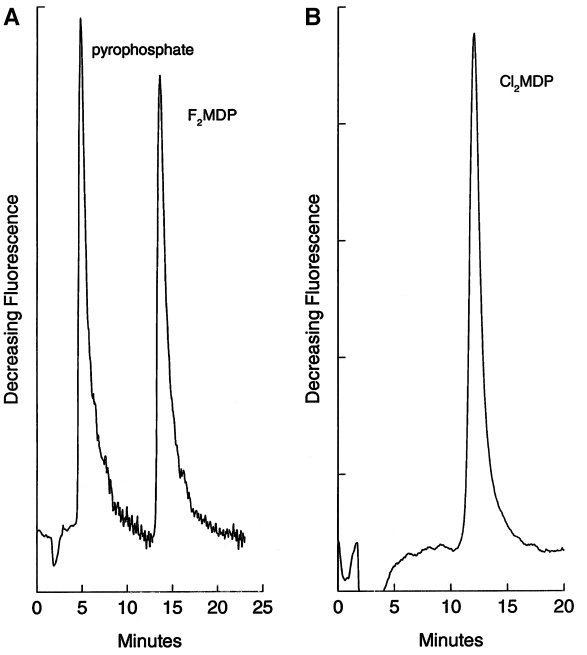


Fig. 3. Chromatograms for the separation and IFD of (A) pyrophosphate and F_2MDP standards and for (B) a Cl_2MDP standard. (A) A PRP-X100 anion-exchange column and an aqueous 40 m*M* $NaNO_3$, 4.1 m*M* NaOH mobile phase at 1.0 ml/min; the post-column reagent for IFD is 2 μ*M* Al^{3+}, 12 μM morin, 37 m*M* $HOAc–OAc^-$, 80% EtOH delivered at 1.0 ml/min with a 650 μl reaction coil volume at 65°C and (B) the same as in (A) except at 80°C.

lyzed in concentrated HCl for about 24 h at about 90°C. The hydrolysate was then evaporated to a few drops of liquid, neutralized with sodium acetate, filtered, diluted to a small known volume, and 10 to 500 μl of sample was injected. The sample matrix components, which remained in the sample and were also injected in the sample aliquot, did not interfere in the separation or in the detection of Cl_2MDP by IFD.

Retention time for the bisphosphonate analytes on the PRP-X100 anion exchanger for an aqueous 40 m*M* $NaNO_3$, 4.1 m*M* NaOH mobile phase is EHDP$<Cl_2$MDP$<F_2$MDP. This corresponds to bisphosphonate acidity where F_2MDP is the strongest acid [3]. Fig. 4A illustrates the separation of 625 pmol Cl_2MDP and 788 pmol F_2MDP standards using the aqueous 40 m*M* $NaNO_3$, 4.1 m*M* NaOH mobile phase. When Cl_2MDP was used as an internal standard for a F_2MDP calibration curve the linear relationship was defined by the equation, peak area ratio of $F_2MDP/Cl_2MDP = 1.1345 \cdot 10^{-3}$ pmol $F_2MDP - 0.021510$ with an r value of 0.9965. The detection limit for F_2MDP at $S/N = 3$ was 4 ng.

Fig. 4B illustrates the chromatogram for the determination of F_2MDP in a cell growth media solution, which was found to be 0.01 m*M* F_2MDP, that was used for the incubation of embryonic rat bones. The Cl_2MDP was added as an internal standard. In the procedure a known aliquot of the growth media–F_2MDP sample was combined with a known amount of the internal standard and diluted by a factor of 10, filtered, and 10 μl of the solution was injected. F_2MDP was also determined by the same internal standard procedure in rat bone and chicken cell samples equilibrated with the cell growth media.

A direct calibration procedure based on a linear relationship between F_2MDP peak area and amount of F_2MDP standard was also used for the determination of F_2MDP in embryonic rat bones that had been equilibrated with culture medium containing F_2MDP. Either equilibration time or amount of F_2MDP equilibrated were varied in these studies. Fig. 5 compares the chromatogram found for a F_2MDP standard (Fig. 5A) to a typical chromatogram for the determination of F_2MDP in the equilibrated embryonic bone sample (Fig. 5B). The sampling procedure was similar to the Cl_2MDP sampling procedure outlined previously except that the sample solution was passed through a strong cation-exchange membrane to reduce Ca^{2+} concentration prior to injection into the anion-exchange column. In the equilibration time study the amount of F_2MDP in the embryonic rat bone increased with equilibration time. For example, at 5 min, 6 h and 48 h the amount of F_2MDP determined to be in the embryonic rat

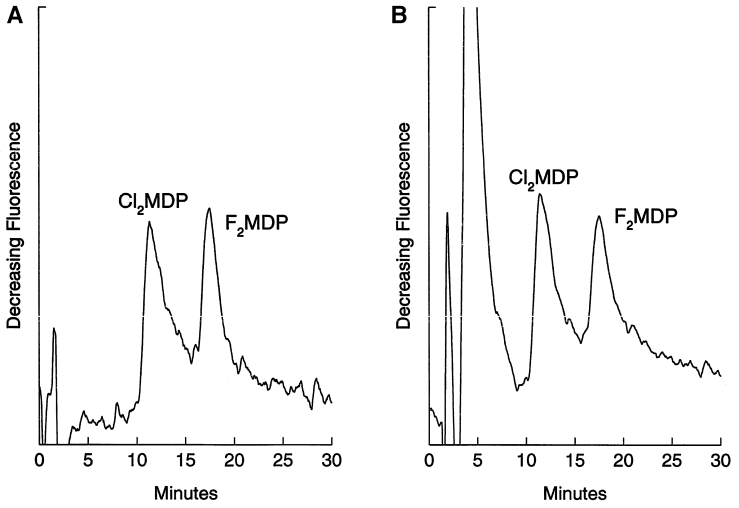


Fig. 4. Chromatograms for the separation and IFD of (A) Cl_2MDP and F_2MDP standards and (B) Cl_2MDP internal standard and F_2MDP analyte in cell growth media. Conditions as in Fig. 3A except that the reaction coil is at 75°C in (A) and 80°C in (B).

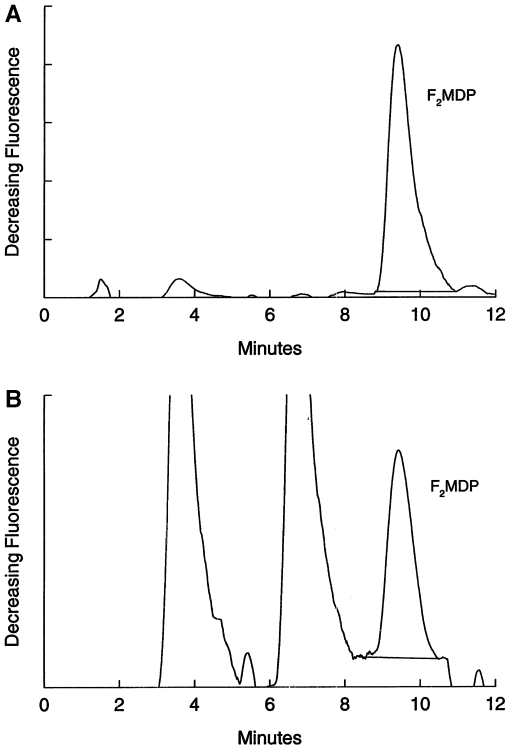


Fig. 5. Chromatograms for the separation and IFD of (A) a F_2MDP standard and (B) F_2MDP analyte in an embryonic rat bone. Conditions as in Fig. 3A except that the mobile phase is 49 m*M* $NaNO_3$, 4 m*M* NaOH and the reaction coil is at 80°C.

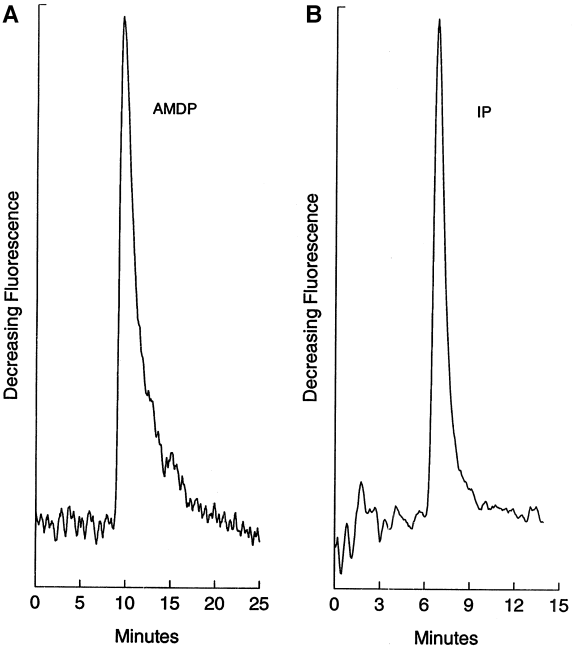


Fig. 6. Chromatograms and IFD for (A) an AMDP standard and (B) a D,L-myo-inositol-1-monophosphate (IP) standard. Conditions as in Fig. 3A except in (A) the mobile phase is ethanol–water (15:85), 30 m*M* $NaNO_3$, pH 9.5 and the reaction coil is 700 μl at 50°C and in (B) the mobile phase is aqueous 10 m*M* $NaNO_3$, pH 9.5 and the reaction coil is 600 μl at 75°C.

bones was 0.56, 20 and 24 μg/mg of bone, respectively, for an average of six measurements.

AMDP is highly retained on the PRP-X100 anion-exchange column and can be detected with sensitivity by IFD after elution from the column. Fig. 6A illustrates the chromatogram obtained for the injection of 650 ng of an AMDP standard using IFD for analyte detection. For the strong mobile phase condition used in Fig. 6A AMDP elutes several minutes prior to F_2MDP if F_2MDP is also in the sample. A calibration curve for AMDP of peak area versus concentration of AMDP standard was linear and was defined by the equation, AMDP peak area = 2578.9 nmol AMDP + 2610.2 with an r value of 0.9981; the upper limit of linearity was not determined. Detection limit for AMDP for $S/N = 3$ was 10 ng of AMDP.

Anion-exchange separation and detection by IFD can be applied to monophosphonic acid derivatives [29,31]. In general these derivatives are less retained than the bisphosphonates on the anion-exchange column and thus a weaker eluent is usually used for their elution. Fig. 6B illustrates the chromatogram with IFD for the elution of an inositol monophosphate derivative on an anion-exchange column using an aqueous pH 9.5, 10 m*M* $NaNO_3$ mobile phase. Inositol phosphates, which are important brain metabolites, are not easily detected after separation except by conductivity or by radiolabeling techniques [35]. Detection by IFD after separation on an anion-exchange column provides a calibration curve with good sensitivity and a wide linear dynamic range. For the D,L-myo-inositol-1-monophosphate (IP) derivative in Fig. 6B, which elutes before phosphate anion, a linear calibration curve was obtained for the injection of 2 to 21 nmol of IP and corresponded to the equation, IP peak area = 37 888 nmol IP + 20 825 with an r value of 0.9995. The

upper limit of linearity was not determined and the detection limit was 210 pmol for $S/N=3$.

4. Conclusions

The Al^{3+}–morin complex is useful as a post-column reagent for the indirect fluorescent detection of bisphosphonates and other analytes including fluoride, pyrophosphate, organic phosphonates and organophosphates such as glyphosate and inositol phosphate derivatives. Post-column indirect detection with absorbance or fluorescence change should also be feasible with other complexes where competitive reactions involving analytes are possible. IFD with the Al^{3+}–morin reagent is reproducible, provides a favorable linear dynamic calibration range, is sensitive, and is selective. For the bisphosphonate analytes detection limit is about 4 to 6 ng of injected analyte depending on the bisphosphonate for $S/N=3$. Selectivity is enhanced by two factors. First, only those analytes that compete with morin to form an Al^{3+} complex are detected. Second, the retention of the bisphosphonate on an anion-exchange column allows its separation from other less retained analyte anions, including phosphate and pyrophosphate as well as sample matrix components that are found in biological samples.

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