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DETERMINATION OF (DICHLOROMETHYLENE)DIPHOSPHONATE IN PHYSIOLOGICAL FLUIDS BY ION-EXCHANGE CHROMATOGRAPHY WITH PHOSPHORUS-SELECTIVE DETECTION

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SUMMARY

An analytical method is presented for the determination of (dichloromethylene)disphosphonate (Cl_2MDP) in serum and urine. Cl_2MDP is isolated from biological samples by adsorption onto precipitated calcium phosphate. Orthophosphate is separated from Cl_2MDP by anion-exchange chromatography using AG 1-X8 resin. Detection is accomplished on-line using a flame photometric detector. Potentially interfering condensed phosphates are removed by acid hydrolysis. Sample handling losses are corrected by monitoring the recovery of a [^{14}C]Cl_2MDP spike added to the samples. Determinations of Cl_2MDP to concentrations as low as 2 μmol/l are possible. Extension of the method to determine other diphosphonates is discussed.

INTRODUCTION

Geminal diphosphonates are chemically similar to pyrophosphate, a substance found in urine and plasma which is able to inhibit the deposition of calcium phosphate salts both in vivo and in vitro [1]. Several calcium metabolic disorders have been linked to abnormally low levels of pyrophosphate in biological fluids [2]. Attempts to correct this imbalance with exogenous pyrophosphate have been unsuccessful presumably due to the rapid chemical and enzymatic hydrolysis of the P—O—P bond [3]. The geminal diphosphonates, possessing a P—C—P rather than a P—O—P linkage, are stable toward chemical and enzymatic hydrolysis and are being investigated as agents for treating certain calcium disorders [4].

The sodium salts of (1-hydroxyethylidene)diphosphonic acid (HEDP) and (dichloromethylene)diphosphonic acid (Cl_2MDP) are two of the more widely

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investigated diphosphonates. A method for the determination of HEDP in serum and urine has been reported which takes advantage of the photolytic sensitivity of HEDP [5]. Cl_2MDP is more resistant to photolysis, and therefore, a more generally applicable diphosphonate method has been developed.

An automated method for Cl_2MDP was sought for numerous serum and urine samples generated by animal studies and human clinical trials. Application of the method over a large concentration range was necessary. The Cl_2MDP concentration varied appreciably (2—25,000 μmol/l) because dosage, route of administration, species, sample type, sample volume, and sample collection time were among the variables.

Analysis of serum was the most challenging application because the concentration of Cl_2MDP following oral administration is low due to poor intestinal absorption, rapid uptake by the skeleton, and efficient clearance by the kidneys. The required detection limit for serum samples of 5 ml volume was 2 μmol/l. Pyrophosphate and orthophosphate are potential interferences that occur in appreciable concentrations in serum and urine. A selective procedure free from such interferences was needed. To meet these requirements, an automated ion-exchange chromatographic procedure for Cl_2MDP similar to a method for pyrophosphate [6] has been developed. Orthophosphate is separated from Cl_2MDP chromatographically and interference from pyrophosphate is eliminated by prior hydrolysis to yield orthophosphate.

Many diphosphonates, including HEDP and Cl_2MDP, lack the functional groups readily detected with conventional liquid chromatographic detectors. To allow automation of the chromatographic procedure, a (single-)flame photometric detector similar to that of Julin et al. [7] was built and used for dynamic detection of diphosphonate in the chromatographic effluent via light emission of HPO. In designing this detector the sources and types of noises leading to measurement uncertainties were considered and their influences minimized.

EXPERIMENTAL

Apparatus

The liquid chromatograph shown schematically in Fig. 1 was assembled from components available commercially. The use of hydrochloric acid as eluent restricted the choice of chromatographic equipment to components with glass, PTFE, Tefzel and Kel F eluent contact points. A Cheminert Metering Pump CMP-2VK (Laboratory Data Control, Riviera Beach, FL, U.S.A.) was used to deliver the 0.025 *M* HCl (Eluent A) at a constant flow-rate. The second, stronger eluent, 1.0 *M* HCl (Eluent B), was loaded into a 10-ml loop of an automatic slider valve No. SV 8031 (Laboratory Data Control) from a reservoir No. 1108PF with Tefzel valves and keys (Omnifit, Cedarhurst, NY, U.S.A.) pressurized with a Pressure Stat No. 050 PBS (Omnifit). Individual samples were loaded into a 20-loop rotary sample injection valve No. ROSV-1.0 controlled by a Valve Drive Unit VDU-20 (Laboratory Data Control). A cam programmer No. 324C-06-F2D-R1A-O1X with an 18-min cycle time (Automatic Timing and Controls, King of Prussia, PA, U.S.A.) indexed the valve drive for automatic injection of samples and controlled solenoids No. 902-00 (Altex Scientific,

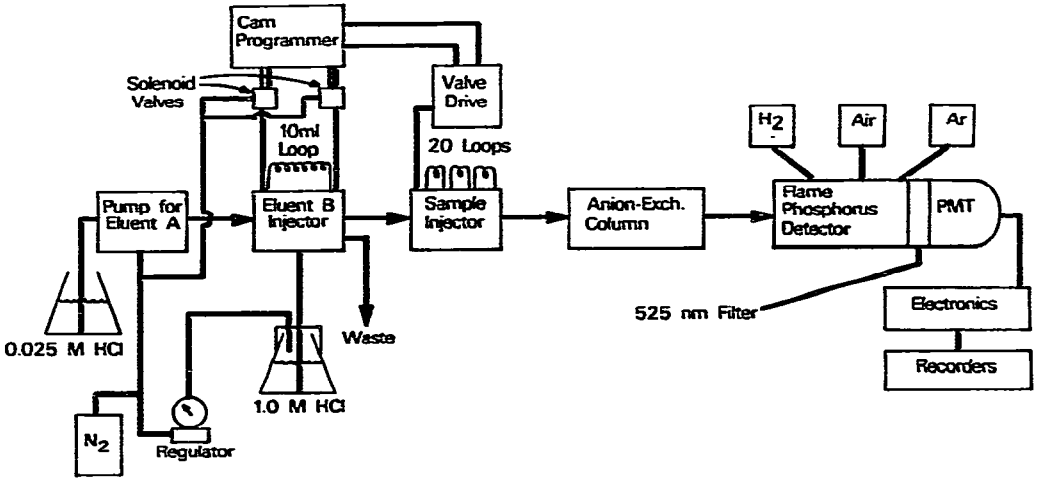

Fig. 1. Schematic diagram of the liquid chromatograph.

Berkeley, CA, U.S.A.). The solenoids were used to switch PA 875 pneumatic actuators (Laboratory Data Control) of the slider valve used for delivery of the second eluent. The chromatographic column No. 252-00-501 (Altex Scientific) was modified from the standard 250 mm length to approximately 100 mm. The column was packed with AG 1-X8 anion-exchange resin, 100—200 mesh (Bio-Rad Labs., Richmond, CA, U.S.A.).

The flame phosphorus detector is shown schematically in Fig. 2. Most of the components are available commercially. The total chromatographic effluent is introduced into a nebulizer No. 303-0352 and burner assembly No. 290-0358 (Perkin-Elmer, Norwalk, CT, U.S.A.) fitted with a burner head constructed by the Miami Valley Laboratories Machine Shop. The burner head design is similar to one described by Haraguchi and Winefordner [8] except that no capillaries are used. Instead, the flame is burned on the top of the inner cylinder. A hydrogen—argon—air entrained flame is used. Argon is applied to the nebulizer at 14 l/min, hydrogen is admitted through the fuel port at 3.6 l/min and air is applied to the sheath attachment at 7 l/min using standard flow controllers and rotameters (Brooks Instrument Division, Emerison Electric Co., Hatfield, PA, U.S.A.). The burner head is covered with a cylindrical sheath which also contains the photometer.

A 1 X 1 in. (2.5 cm) three-cavity interference filter with a center wavelength of 525.0 nm and a 4.0-nm bandpass (Ditric Optics, Marlboro, MA, U.S.A.) serves to isolate the HPO emission. A Model 83-021 Photomultiplier Housing (Jarrell-Ash Div., Fisher Scientific, Waltham, MA, U.S.A.) was used for either a type R106 photomultiplier tube (Hamamatsu Corp., Middlesex, NJ, U.S.A.) or a type 1P28A photomultiplier tube (Radio Corporation of America, Harrison, NJ, U.S.A.). The photomultiplier tube is connected to a Model 244 high-voltage supply and a Model 427 current amplifier (Keithley Instruments, Cleveland, OH, U.S.A.). A variable attenuator and a passive RC filter (time constant = 5 sec) constructed from standard electronic components are used to range and smooth the signal.

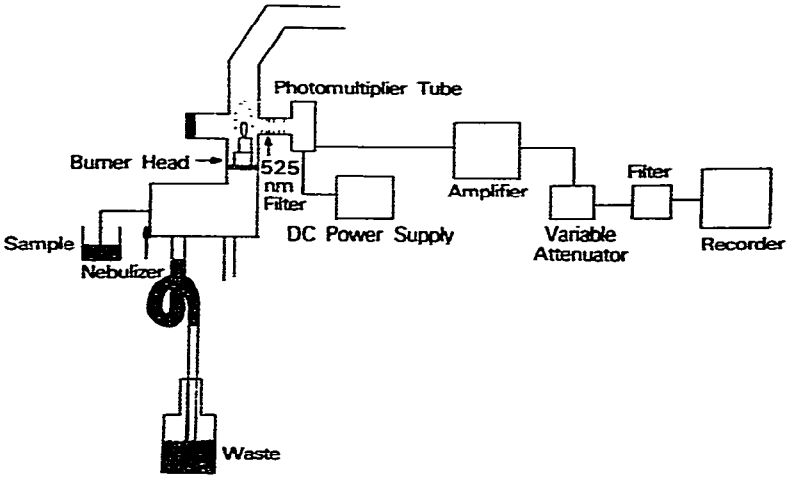

Fig. 2. Schematic diagram of the flame photometric detector.

Chromatograms are recorded using a Model 7132 strip chart recorder (Hewlett-Packard, Dayton, OH, U.S.A.).

Reagents and solutions

Test solutions of phosphoric acid and Cl_2MDP (acid form) were prepared to check detector performance. Test solutions of phosphoric acid were prepared by dilution of Baker (Phillipsburg, NJ, U.S.A.) reagent grade 85% phosphoric acid. Dichloromethane diphosphonate from Procter & Gamble (Cincinnati, OH, U.S.A.) was converted to the acid form by ion exchange on AG 50W-X8 resin (Bio-Rad Labs.) before preparation of less concentrated solutions of Cl_2MDP by dilution. Stock solutions of [^{14}C] Na_2Cl_2MDP (5 μCi/ml) were provided by the Miami Valley Laboratories Radiochemistry Department for use as internal recovery standard. EDTA diluent was prepared by dilution of 1 part of a saturated solution of reagent grade disodium (ethylene-dinitrilo)-tetraacetic acid with 9 parts of water.

Eluent A (0.025 *M* hydrochloric acid) and Eluent B (1.0 *M* hydrochloric acid) were prepared by dilution of Baker reagent grade concentrated hydrochloric acid.

All other chemicals were reagent grade.

Sample preparation

The sample preparation procedure for Cl_2MDP determination is similar to that of Bisaz et al. [5] for HEDP in urine and plasma.

An internal standard of approximately 0.05 μCi (5 μg) of [^{14}C] Cl_2MDP is added to each of the samples and allowed to equilibrate before preparation. Samples are then deproteinized with 25% trichloroacetic acid (4 ml added per 5 ml of serum or 1 ml for 5 ml of urine). Deproteinized serum and urine are then prepared similarly. To the protein-free filtrate, 100 μl of 0.5 *M* sodium dihydrogen phosphate and 50 μl of 2.5 *M* calcium chloride are added, and the pH is adjusted to 12.0—12.5 with 25% sodium hydroxide. The resulting precipitate is isolated by centrifugation, washed with deionized water, and redissolved

in 5 ml of 2 *M* hydrochloric acid. This solution is then heated for 30 min on a boiling water bath to hydrolyze any pyrophosphate present to orthophosphate. The pH is then adjusted to 7.0 using first 25% sodium hydroxide and finally 2.5% sodium hydroxide. Again a precipitate forms that is separated by centrifugation and is then dissolved in 2.0 ml of EDTA diluent. An aliquot of 100 μl is removed for determination of recovery of [^{14}C]Cl_2MDP by scintillation counting on a Model 2450 (Packard Instrument, Downers Grove, PA, U.S.A.). This is done to correct for losses occurring in the sample preparation. Aliquots are taken from the remainder for chromatographic analysis.

Standards are prepared by adding known quantities of Na_2Cl_2MDP and [^{14}C]-Na_2Cl_2MDP to blank urine or serum samples. These matrix standards are processed by the same procedure as used for samples. Quantitation of the eluted Cl_2MDP is accomplished by peak height measurement.

Chromatography

Samples, standards and controls are loaded into 1.0-ml loops attached to the 20-port valve. Samples are injected onto the column with Eluent A flowing. The Eluent B loop is bypassed for approximately 10 min, then is injected onto the column to elute the Cl_2MDP. The Eluent B loop is automatically loaded during the Eluent A cycle from a pressurized reservoir containing 1.0 *M* hydrochloric acid.

RESULTS

Detector performance

Aqueous standards of H_3PO_4 and Cl_2MDP (acid form) were directly aspirated into the detector to determine the dynamic range of the detector free of

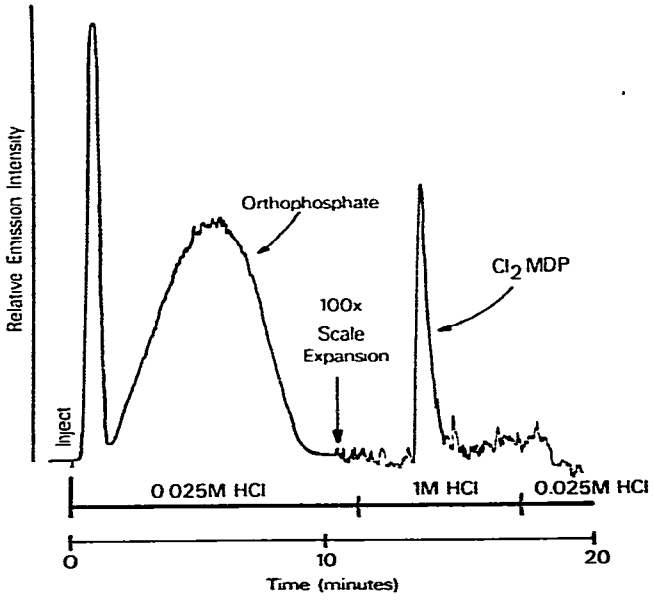

Fig. 3. Chromatogram of serum sample containing 10 μg of Na_2Cl_2MDP.

chromatographic constraints. The phosphorus detector behaved linearly for both compounds over the range of 0.1 μmol/l (0.003 mg phosphorus per liter, the detection limit) to approximately 3200 μmol/l (100 mg phosphorus per liter). Absolute response to phosphorus was approximately equal for these compounds. Response factors may vary for other analytes containing phosphorus, and, for chromatographic conditions, response factors will also depend on chromatographic variables.

Detector performance is severely degraded when solvents or chromatographic eluents other than deionized water or hydrochloric acid are used. However, hydrochloric acid is a suitable eluent for this chromatographic application. The small change in the baseline signal when changing from Eluent A to Eluent B is hardly noticeable in Fig. 3 and is quite acceptable for determination of Cl_2MDP. This small baseline change may reflect the difference in trace impurity level in the two eluents.

Validation of the method

Daily controls prepared by adding known amounts of Cl_2MDP to blank serum are analyzed with each group of samples. Validation data for these human serum controls are given in Table I to demonstrate the sustained accuracy and precision to be expected. Determinations are reliable (with relative standard deviations less than 10%) to concentrations as low as 2 μmol/l when 5 ml of serum are taken for analysis. For best accuracy, samples near this limit should be bracketed in concentration by standards. This limit is adequate for determination of serum profiles (0—10 h) following single oral doses of 3200 mg or 1600 mg Cl_2MDP. For oral doses less than 1600 mg of Cl_2MDP the resulting Cl_2MDP serum concentration is usually less than 2 μmol/l. Thus, the volume of serum required for reliable analysis becomes prohibitive (that is, greater than 5 ml).

A sample chromatogram is given in Fig. 3 showing the detector response for an injection of 10 μg (0.035 μmol) of Cl_2MDP.

Urinary determinations are especially valuable when lower oral doses are administered or when sufficient serum is unavailable. Validation data for

TABLE I

SUMMARY OF DAY-TO-DAY PRECISION AND ACCURACY FOR DETERMINATION OF Cl_2MDP IN HUMAN SERUM

Number of standards	Cl_2MDP (μmol/l)			S.D. (μmol/l)	R.S.D. (%)
	Added	Mean found	Difference		
7	3.46	4.74	1.28	0.26	5.5
5	10.4	9.62	−0.78	0.62	6.4
7	17.3	17.2	−0.1	0.69	4.0
7	24.2	26.4	2.2	2.1	8.0
3	69.2	67.8	−1.4	—	—
1	103.8	97.2	−6.6	—	—
1	138.4	133.2	−5.2	—	—

human urine are given in Table II. The precision for these blank samples spiked with Cl_2MDP adequately represents the agreement found for actual samples analyzed in duplicate. If chromatographic fractions are collected, wet-ashed with perchloric acid, and assayed colorimetrically for phosphorus [9], similar precision is obtained.

TABLE II

SUMMARY OF DAY-TO-DAY PRECISION AND ACCURACY FOR DETERMINATION OF Cl_2MDP IN HUMAN URINE

Number of standards	Cl_2MDP (μmol/l)			S.D. (μmol/l)	R.S.D. (%)
	Added	Mean found	Difference		
2	17.3	14.7	−2.6	—	—
2	34.6	34.1	−0.5	—	—
2	69.2	68.5	−0.7	—	—
2	103.8	110.0	6.2	—	—
1	115.2	109.0	−6.2	—	—
8	138.4	138.8	0.4	9.7	7.0
8	173.0	173.1	0.1	7.2	4.2
5	230.8	235.5	4.7	16	6.7

DISCUSSION

Detector design considerations

The most prevalent source of uncertainty (or noise) in this detector is the flickering of the flame. The photometric signal, which is due to the flame background light plus the light emission of HPO, changes as the flame moves around. Since flicker noise is directly proportional to its light flux carrier [10], collecting additional light with a mirror would increase both the signal and noise by the same factor. Since no net improvement in signal-to-noise ratio or relative measurement uncertainty would result, we omitted the light-gathering mirror used by other workers [7, 11].

The flame background is a near-continuum at 526 nm. Thus, a filter was chosen with a bandpass approximately equal to the width of the major peak in the HPO spectrum in order to maximize the signal-to-background ratio (and thus the signal-to-flicker noise ratio) [12]. This filter also had sufficient transmittance to keep photon noise and photomultiplier shot noise (which are proportional to the square root of the light flux and dark current, respectively) small compared to the flame background flicker noise.

The relatively inexpensive photomultiplier tube (RCA 1P28A) is perfectly adequate for this application since the limiting noise is carried on the flame background light. Switching to the photomultiplier tube (Hamamatsu R106) with lower relative dark current had no effect on the detector performance.

Chromatography

Small amounts of Cl_2MDP must be separated from much larger amounts of calcium phosphate salts and EDTA by the chromatographic procedure. The

instantaneous eluent upon injection of a 1.0-ml sample is not Eluent A but the sample diluent. The peak heights of aqueous Cl_2MDP standards do not adequately reflect the actual Cl_2MDP concentration due to broadening of the Cl_2MDP band on the column in the presence of this diluent. To eliminate low bias, we chose to standardize the method by the use of standards prepared in blank serum or urine matrix and processed by the same procedure as the samples. Matrix standard curves are prepared for each group of samples by plotting the mass of Cl_2MDP injected versus the peak height. The amount injected for each sample is calculated by a least squares linear regression of the matrix standards. The linear regression equation generally gives a negative intercept on the peak height axis indicating slight curvature in the calibration curve near the detection limit. The intercept is not eliminated by subtraction of the baseline signal change which accompanies the step gradient.

We have experience with chromatographic columns having smaller diameters but sufficient capacity to retain aqueous solutions of Cl_2MDP. The smallest detectable amount of Cl_2MDP was reduced from 7 nmol (2 μg) to 0.7 nmol (0.2 μg) injected, and a zero intercept was observed using these columns; however, the capacity was insufficient for actual samples. The recovery of Cl_2MDP in the chromatographic step is essentially quantitative for actual samples if the recommended column is used. Recovery of Cl_2MDP in the chromatographic step of the procedure was severely degraded when the smaller columns were tried for actual samples. A chromatographic peak of 7 nmol of Cl_2MDP represents the detection limit of the method because lesser amounts are indistinguishable from the background due to increased band spreading for the wider chromatographic column.

The use of more efficient chromatographic supports and stainless-steel columns is restricted by the use of hydrochloric acid eluents compatible with this flame phosphorus detector. A more versatile flame phosphorus detector for liquid chromatography has now been developed that can be used with many organic eluents and with ion-pairing reagents [13, 14].

We have successfully used this method for determination of Cl_2MDP in more than 3000 serum and urine specimens of animal and human origin. Generally 13—15 samples plus 4 or 5 standards are prepared the day prior to the chromatographic analysis. The operation of the chromatograph is completely unattended once the sample loops of the injector are loaded. The cycle time (18 min) and injector capacity (19 samples) are suitable for operation during a normal working day. We prefer to operate the flame phosphorus detector during normal working hours even though its operation has proven to be safe for the past two years. The capacity of the method can be more than doubled by using a single detector to dynamically monitor only the Cl_2MDP peaks from two chromatographs that are one-half cycle out of phase with one another. The orthophosphate from each column is then vented to waste using a slider valve to switch between column effluents.

The capacity of the method also can be increased by automated and unattended collection of the Cl_2MDP fractions during the evening. The Cl_2MDP fractions can then be loaded into an automatic sampler for direct aspiration into the detector the following morning. Oftentimes the range of Cl_2MDP concentrations is greater than the range of the standard curve. Dynamic detection

destroys the entire sample so those samples falling outside the range of the standard curve must be prepared again on a new dilution. This difficulty is circumvented by this alternative procedure because the entire sample need not be consumed: further dilutions of Cl_2MDP fractions can still be prepared. Another advantage is that the same group of standards can be used for more samples than is possible with dynamic detection.

Collection of Cl_2MDP fractions for a colorimetric total phosphorus determination after wet ashing with perchloric acid offers an alternative method for serum and urine samples when a flame phosphorus detector is unavailable.

Other diphosphonates

The method described here offers a general method for diphosphonates in physiological samples. Preliminary work has shown that the concentration of Eluent A should be decreased to 0.01 *M* hydrochloric acid for HEDP and (methylene)diphosphonate (MDP). An alternative diluent of 0.03 *M* hydrochloric acid will redissolve the final precipitate without introducing a reagent blank from EDTA that accompanies this change in the first eluent. The amount of resin must also be increased to prevent premature elution of some diphosphonates. With these modifications the application of the method to determine diphosphonates in physiological samples is straightforward. Determination of pyrophosphate in physiological samples is also a potential application with omission of the acid hydrolysis step. The cycle time must be increased to 40—45 min for HEDP and MDP determinations because the orthophosphate elution is extended with these modifications. Fraction collection with subsequent off-line detection is preferable when many samples must be analyzed at longer cycle times.

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