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The determination of 4-amino-1-hydroxybutane-1,1-diphosphonic acid monosodium salt trihydrate in pharmaceutical dosage forms by high-performance liquid chromatography*

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Abstract: A rapid, sensitive and specific high-performance liquid chromatographic (HPLC) assay is reported for the determination of 4-amino-1-hydroxybutane-1,1-diphosphonic acid (AHBuDP) monosodium salt trihydrate, a new inhibitor of bone resorption. The compound does not demonstrate any intrinsic UV properties and thus pre-column derivatization of the primary amino group of the drug with 9-fluorenylmethyl chloroformate (FMOC) at pH 9 in the presence of sodium citrate is required to facilitate UV detection of the analyte. Excess derivatization reagent is extracted with methylene chloride and an aliquot of the aqueous portion is assayed on a polymeric phase (Hamilton PRP-1) at 35°C by reversed-phase HPLC. A mobile phase of 0.05 M citrate and 0.05 M phosphate buffer (pH 8.0)–acetonitrile–methanol (75:20:5, v/v/v) is utilized with UV detection at 266 nm. Application of the method to the analysis of AHBuDP in I.V. solution, tablet and capsule formulations is presented.

Keywords: *Diphosphonate; HPLC analysis; FMOC derivatization; pharmaceutical analysis; AHBuDP.*

Introduction

4-Amino-1-hydroxy-butane-1,1-diphosphonic acid (AHBuDP; Fig. 1) monosodium salt trihydrate, is a new member of the diphosphonate class of compounds being investigated as inhibitors of bone resorption. The direct chromatographic analysis of this class of compounds is complicated due to the lack of a suitable UV chromophore for conventional high-performance liquid chromatographic (HPLC) analysis and insufficient volatility for gas chromatographic analysis.

Etidronate disodium [1-hydroxyethylidene-1,1-disodium phosphonate (HEDP) disodium salt], a drug presently utilized in the treatment of metabolic bone diseases, has been assayed as its free acid by gas chromatography with flame ionization detection via the formation of trimethylsilyl (TMS) [1, 2] and trifluoroacetyl ester derivatives [3]. The

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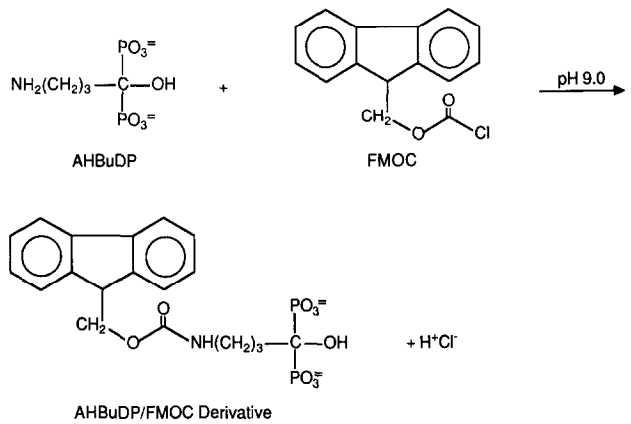

Figure 1
Derivatization reaction between AHBuDP and FMOC.

method of Ismail *et al.* [2] was validated for the quantitation of etidronate disodium in tablets. A non-chromatographic assay utilizing titration with thorium diaminocyclohexanetetraacetate with a colorimetric endpoint [4] has been reported for etidronate disodium in tablets [5]. Recently, HPLC analysis of etidronate disodium in tablets was described utilizing an ion-exchange separation followed by element specific detection of phosphorous with an inductively coupled plasma (ICP) detector [6]. Although requiring instrumentation not typically available in the routine pharmaceutical analysis laboratory, the assay demonstrated excellent accuracy, precision and linearity for the drug in 200 mg tablets.

Assays in physiological fluids also have been reported for the sodium salts of dichloromethylene diphosphonate (Cl_2MDP) by ion-exchange followed by on-line flame photometric detection [7] and 4-chlorophenylthiomethylene bisphosphonic acid by ion-pair reversed-phase HPLC with UV detection [8]. The latter compound is the only member of this class which is reported to possess a sufficiently sensitive chromophore to make direct analysis a viable option without resorting to specialized equipment or derivatization. Additionally, the feasibility of ion-exchange chromatography with post-column reaction (formation of aluminium–morin complex) and indirect fluorometric detection has been demonstrated for a series of sodium salts of disphosphonates including difluromethylene diphosphonate (F_2MDP), Cl_2MDP and HEDP [9].

Direct analysis of AHBuDP via chromatographic assay also is not feasible due to the lack of volatility and suitable chromophore. However, the molecule does contain a primary amino group which can be readily derivatized with a number of reagents to form a compound with chromophoric properties thus facilitating HPLC analysis. A HPLC post-column derivatization procedure has been reported for AHBuDP using orthophthaldehyde (OPA) and ion-paired reversed-phase chromatography with fluorescence detection [10]. This procedure requires specialized equipment consisting of a knitted post-column reactor and associated pumping systems which are inconvenient for routine applications.

A pre-column HPLC procedure is now reported for the assay of AHBuDP utilizing 9-fluorenylmethyl chloroformate (FMOC) derivatization. This reagent rapidly reacts with the drug at ambient temperature to yield a stable derivative with suitable chromato-

graphic and UV properties which enable facile assay by conventional reversed-phase HPLC.

Experimental

Column

The column used was a 250 × 4.1 mm, i.d., stainless steel prepacked column containing 10 μm PRP-1 (Hamilton, Reno, NV, USA).

Instrumental parameters

The HPLC system consisted of a Perkin–Elmer Model 410 (Norwalk, CT, USA) with an ABI 783 variable wavelength detector (Ramsey, NJ, USA).

The isocratic mobile phase was a mixture of 0.05 M sodium citrate 0.05 M potassium phosphate buffer (pH 8.0)–acetonitrile–methanol (75:20:5, v/v) delivered at a flow rate of 1.0 ml min^{-1} at 35°C. Under these conditions, the retention time of the AHBuDP/FMOC derivative was approximately 7 min ($K' \simeq 4.0$). Detection was by UV at 266 nm, the absorption maximum of the FMOC derivative. Chromatograms were recorded on a Model SP 4270 integrator (Spectra-Physics, Santa Clara, CA, USA). A typical chromatogram for the AHBuDP/FMOC derivative (1.25 μg equivalent of free acid/50 μl injection) is shown in Fig. 2.

Dissolution assays were performed utilizing USP Method II (paddles) at an agitation speed of 50 rpm in 900 ml deionized water thermostatically controlled to 37 ± 0.1°C.

Analytical standard

AHBuDP monosodium salt trihydrate (MK-217, $C_4H_{12}NNaO_7P_2 \cdot 3H_2O$, M_W 325.13) of pharmaceutical grade purity (>99%), manufactured within Merck Sharp & Dohme Research Laboratories, was used as an analytical standard.

Standard solutions

(1) *Potency and uniformity assay*. About 65.3 mg of AHBuDP monosodium salt trihydrate was accurately weighed into a 200-ml volumetric flask and diluted to volume

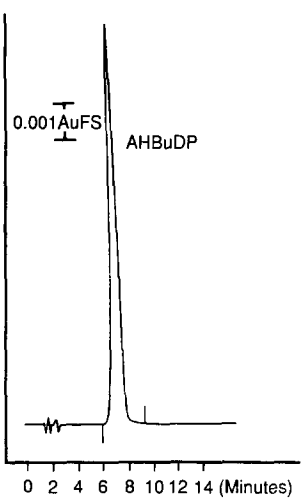

Figure 2
Chromatogram of AHBuDP/FMOC derivative. HPLC conditions were as follows. Column: Hamilton PRP-1, 250 × 4.1 mm, i.d.; 10 μm. Mobile phase: 0.05 M phosphate and 0.05 M citrate (pH 8.0)–acetonitrile–methanol (75:20:5, v/v/v). Flow rate: 1.0 ml min^{-1}. Temperature: 35°C. Detection: UV @ 266 nm. Amount injected: 1.25 μg/50 μl.

with 0.1 M sodium citrate (stock standard solution). A 10-ml volume of this solution was diluted to 100 ml with 0.1 M sodium citrate (working standard solution). A 5.0-ml aliquot of this standard was placed into a 50 ml polypropylene centrifuge tube.

(2) *Dissolution assay*. About 43 mg of AHBuDP monosodium salt trihydrate was accurately weighed into a 500-ml volumetric flask and diluted to volume with water (dissolution stock standard solution). Aliquots of this stock were diluted with water to yield concentrations equivalent to those expected for the dissolution of 2.5–60 mg dosage formulations in 900 ml of dissolution fluid (water). A 5.0-ml aliquot of this standard was placed into a 50-ml polypropylene centrifuge tube.

Reagents

All reagents were of analytical purity and solutions prepared using deionized, distilled water.

(1) *Citrate (0.05 M)/phosphate (0.05 M) buffer*. 14.7 g of sodium citrate dihydrate and 8.7 g of anhydrous potassium phosphate dibasic were dissolved in 900 ml of water, the pH adjusted to 8.0 with phosphoric acid and the solution made up to 1000 ml with water. The solution was filtered through a 0.45-μm membrane filter (Nylon 66 or equivalent) prior to use.

(2) *Sodium borate* (0.1 M). 20.1 g of sodium borate decahydrate dissolved in 1000 ml of water.

(3) *Sodium citrate* (0.1 M). 29.4 g of sodium citrate dihydrate dissolved in 1000 ml of water.

(4) *Sodium citrate* (0.6 M). 44.1 g of sodium citrate dihydrate dissolved in 250 ml of water.

(5) *FMOC reagent* (0.5%). 50 mg of 9-fluorenylmethyl chloroformate (Aldrich Chemical Co. Inc., USA) dissolved in 100 ml of acetonitrile. A fresh batch of this reagent should be prepared at the beginning of each week and kept refrigerated.

Organic solvents include methanol, methylene chloride and acetonitrile (HPLC grade). HPLC mobile phase: 0.05 M citrate and 0.05 M phosphate buffer (pH 8.0)–acetonitrile–methanol (75:20:5, v/v/v).

Assay procedure: (A) sample preparation

(1) *I.V. Solutions*. These are diluted to the assay concentration of 25 μg ml^{-1} with 0.1 M sodium citrate. A 5.0-ml aliquot of this solution is transferred to a 50-ml polypropylene centrifuge tube.

(2) *Capsules*. These are opened and the entire contents of one (uniformity assay) or 10 (composite assay) capsules are placed into 100 or 1000 ml volumetric flasks, respectively. The flasks are half-filled with 0.1 M sodium citrate, and drug dissolution is accomplished via automatic shaking (30 min) followed by ultrasonification (5 min) prior to the flask being brought to volume with 0.1 M sodium citrate. The samples are then diluted to 25 μg ml^{-1} and a 10-ml aliquot is transferred to a 10-ml test tube and centrifuged at 2000

rpm for 10 min. A 5.0-ml aliquot of this sample is placed into a 50-ml polypropylene centrifuge tube.

(3) *Tablets.* Dosage uniformity and composite assay are performed as described for the capsule with the exception that the whole tablets are assayed in place of the capsule contents.

(4) *Dissolution.* Samples (9 ml) are removed from the dissolution apparatus via a 10-ml syringe, fitted with a disposable filter fitted onto the cannula, and placed into a 10-ml test tube. After centrifuging (2000 rpm for 10 min) a 5.0-ml aliquot is transferred to 50 ml polypropylene centrifuge tube to which is then added one (1.0) ml of 0.6 M sodium citrate.

(B) Derivatization procedure and analysis

To the 50 ml centrifuge tube containing the sample or standard in sodium citrate solution, is added 5.0 ml of 0.1 M sodium borate (pH 9.0). After mixing 4.0 ml of the FMOC reagent is added, the tube capped and vortexed for approximately 30 s and the derivatization reaction allowed to proceed for 30 min. At the end of the reaction time 25 ml of methylene chloride was added and the tube shaken for 30–60 s, allowed to stand for 5 min and then centrifuged at 1000 rpm for 5 min to remove excess reagent. A portion of the top aqueous layer was carefully removed and transferred to a HPLC vial.

A 50-μl aliquot of both standards and samples is assayed utilizing the HPLC conditions described previously. The potency of the product is calculated as mg AHBuDP free acid equivalent based upon the area response of the standard correcting for the dilution factors, purity, and molecular weight of the sodium salt trihydrate (the active ingredient in the dosage form). Dissolution results are expressed as percent dissolved of label claim of AHBuDP free acid equivalent utilizing the same corrections described for the potency assays.

Results and Discussion

Pre-column derivatization of AHBuDP by means of reactions with OPA, fluorescamine and FMOC [11] was investigated. The FMOC reagent was selected due to its ability to react rapidly and quantitatively at room temperature and to form a derivative with increased stability. The reaction of AHBuDP with the FMOC requires a 2-fold excess of reagent and a reaction time of at least 20 min (Fig. 3) to ensure a yield of derivative proportional to the concentration of AHBuDP. The derivative was found to be stable for a period in excess of 24 h in polypropylene tubes. Excess derivatization reagent is reported to be extracted into pentane, hexane or any other semi-polar to non-polar solvent [12]. Methylene chloride was selected in this procedure to allow the excess reagent and acetonitrile to partition into the lower phase, thus simplifying removal of the derivative for subsequent HPLC analysis. Failure to apply the extraction procedure resulted in the appearance of assymetric peaks.

The derivative is amenable to UV and fluorescence detection, as illustrated in Figs 4 and 5, respectively. Adequate sensitivity for quantitation is achieved in pharmaceutical dosage forms by UV measurements at 266 nm. The citrate ion in the reaction media is critical for quantitative yields of the derivative. Experiments have demonstrated that the citrate ion acts as a sequestrant of metal ions in solution allowing AHBuDP to exist in a

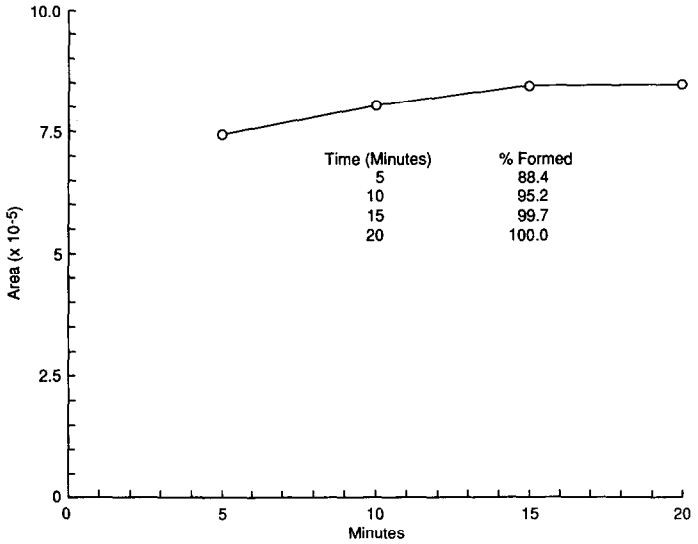

Figure 3
Rate of formation of the AHBuDP/FMOC derivative.

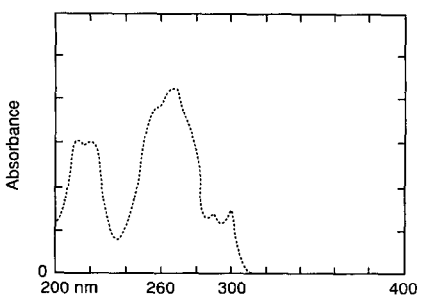

Figure 4
UV spectrum of the AHBuDP/FMOC derivative.

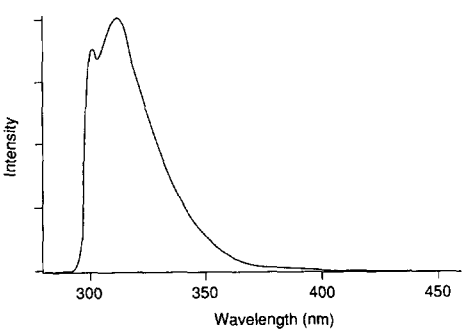

Figure 5
Fluorescence emission spectra of the FMOC derivative of AHBuDP with excitation at 266 nm.

Table 1
Recovery data for AHBuDP/FMOC derivative from spiked placebo tablet extracts in the presence of citrate ion

Citrate reagent molarity (M)	% Recovery
0	82.2
0.025	97.8
0.050	99.3
0.100	99.9

Sample: 2.5 μg AHBuDP/ml.

"non-complexed" form available for derivatization. This phenomenon is clearly illustrated (Table 1) for the analysis of an extract (2.5 μg ml^{-1}) of tablets with varying amounts of citrate ion. EDTA also has been shown to be an efficient sequestrant of metal ions which also will enable the derivatization of the uncomplexed AHBuDP to proceed to completion. For the dissolution assay, the preferred USP solvent of water is utilized as the dissolution medium. The addition of 1 ml of the 0.6 M sodium citrate to the sample provides the proper strength of citrate ion to facilitate quantitative derivatization.

The chromatographic separation is performed on a styrene divinylbenzene copolymer reversed-phase HPLC column in order to enable the use of an alkaline (pH 8.0) mobile phase necessary to ensure derivative stability. Additionally, analysis time is significantly reduced (Rt = 7.4) at pH 8.0 compared with those observed with more acidic mobile phases (Rt = 20.8 at pH 3.5). Attempts to use conventional C8 or C18 reversed-phase silica based columns which have documented instability at these higher pH values met with little success due to their inability to retain the derivative.

The selected mobile phase consists of 0.05 M sodium citrate and 0.05 M sodium phosphate (pH 8.0)–acetonitrile–methanol (75:25:5, v/v/v). It was found that the addition of methanol significantly reduces column equilibration time. The retention of the derivative has been shown to increase with increasing ionic strength and decreasing pH of the mobile phase.

Assay validation

Derivatization of AHBuDP with FMOC yields a AHBuDP product which was found to yield a linear response over a concentration range of 1–100 μg ml^{-1} (r = 0.9999), which encompasses (50–150% of theory) the range of assay concentrations expected for all potency and dissolution assays.

The precision of injection is routinely <1%. The precision of the assay is typically 2% and can vary from 1 to 3% on any given day of analysis. The recovery of the drug obtained by spiking AHBuDP to placebo formulations over an assay concentration encompassing the active concentration is shown in Table 2 for the potency assays. Excellent recoveries and RSD without experimental bias were obtained for each experimental formulation. Similar data were obtained for the dissolution procedure.

AHBuDP has been demonstrated to be stable as a solid (heated at 80°C) for 1 month, or as a solution (refluxed at native pH) for periods exceeding 1 month. To demonstrate the specificity of the assay the compound was heated by DSC to its melt (260°C) and then cooled to room temperature. The resultant sample was derivatized and chromatographically assayed. Approximately 94% of the initial assay value was obtained and a minor degradation peak was observed at 18 min (Fig. 6). Specificity also has been demonstrated

Table 2
Recovery of AHBuDP added to experimental formulations

Dosage form	Potency*	Assay Concentration range	*n*	Recovery	RSD (%)
Injection solution	2.5 mg ml^{-1}	2.00–3.00 mg ml^{-1}	9	99.5	0.8
Capsule	2.5 mg	2.17–3.25 mg	6	101.1	1.5
	60 mg	52.0–78.1 mg	6	100.2	0.8
Tablet I	5 mg	2.50–7.50 mg	3	99.5	0.8
	50 mg	25.0–75.0 mg	3	99.2	1.0

* All potencies expressed in milligrams of free acid.

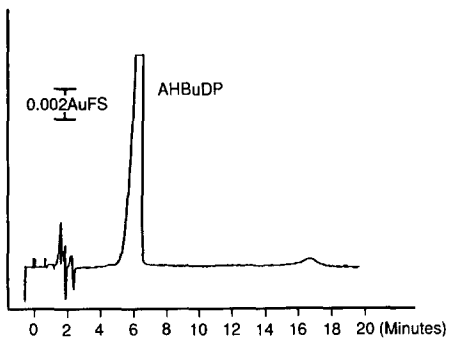

Figure 6
HPLC–UV analysis of FMOC derivative of a degraded sample of AHBuDP formed by DSC heating to melt (260°C).

Table 3
Analysis of pharmaceutical formulations containing AHBuDP

Dosage form	Potency*	*n*	Assay*	Average Claim (%)	RSD (%)
I.V. solution	2.50 mg ml^{-1}	2	2.52, 2.46	99.6	—
	0.25 mg ml^{-1}	2	0.25, 0.26	104.0	—
Capsule	5 mg	10	—	96.8	2.0
	25 mg	10	—	100.8	2.5
	50 mg	10	—	96.8	2.3
Tablet	5 mg	10	—	98.8	1.8
	50 mg	10	—	100.2	1.8

* Expressed as mg of free acid.

in the ability of the mobile phase to separate the FMOC derivatives of AHBuDP/(Rt = 7.4 min) from the amino-hydroxy-propyl analogue (Rt = 6.2 min) and from 4-amino butyric acid, a synthesis precursor (Rt >20 min).

Assay application

The assay was applied to the analysis of AHBuDP in I.V. injection solutions (0.25 and 2.5 mg ml^{-1}), capsules (5–50 mg) and tablets (5 and 50 mg) (Table 3). All measured potencies were >95% of the claimed value. The RSD reported (n = 10) for the solid

formulations (<3% in all cases) represents the sum of the imprecision from the assay and the manufacture of the dosage form. Dissolution data (30 min) for AHBuDP in these capsules and tablet formulations demonstrated acceptable dissolution (>80%).

Conclusion

An HPLC assay for AHBuDP monosodium salt trihydrate utilizing pre-column derivatization with FMOC is reported. The assay has been applied successfully to the analysis of the drug in various dosage forms and for the measurement of drug dissolution rates.

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