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The effect of mobile phase modifiers on the simultaneous LC elution of a gadolinium complex and free ligand

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Abstract: A liquid chromatographic method was developed for simultaneous separation of a gadolinium complex and corresponding ligand using a reversed-phase anion-exchange column. The effect of mobile phase pH, EDTA and chloride ion concentration, added to the mobile phase as counter-ions, on the elution of a metal complex and ligand were investigated. Decreasing the mobile phase pH from 9.4 to 7.4 decreased the retention time of the free ligand but had little effect on changing the retention time of the complex. Increasing the EDTA concentration of the mobile phase from 0 to 0.5 mM decreased the retention of the free ligand but had little effect on changing the retention time of the metal complex. Both the retention times of the metal complex and free ligand decreased as the chloride ion concentration was increased from 0 to 0.2 M.

Keywords: *Anion-exchange chromatography; metal complex; ligand; preformulation; MRI contrast agent; gadolinium.*

Introduction

Paramagnetic metal ions coordinated to a ligand represents a new class of pharmaceutical compounds used as contrasting agents for magnetic resonance imaging (MRI) [1, 2]. These enhancers have been found to increase the delineation of structures in the body, and enhance the image contrast between normal and diseased tissue. Some of these paramagnetic metal ions include Fe (II), Fe (III), Mn (II) and Gd (III). The strength of these metal complexes is determined by their association constants. This is an important consideration in formulation development since certain free metal ions can be toxic. Qualitatively, increasing acidity weakens the metal–ligand complex by protonating the ligand. Hence, when formulating compounds of this type, it may be necessary to control the pH of the formulation at a pH where dissociation of the complex to free ligand and metal ion is at a minimum. Furthermore, certain buffers used in parenteral preparations are sequestering agents (e.g. phosphate) which can complex with free metal ions [3]. Depending on the association constant of the metal complex, this could cause the equilibrium to shift favouring the dissociation of the metal complex. Therefore, during preformulation development, it is convenient to have an HPLC method which will separate both the ligand and chelate. A wealth of information does exist regarding the chromatographic separation of metal ions [4], but few analytical methods have appeared in the literature regarding chromatographic separation of MRI contrasting agents. One previous method for simultaneous separation of a metal complex from the free ligand relied on the use of ion-pair agents [5].

This paper describes the effect mobile phase modifiers (pH, ionic strength and EDTA concentration) have on the elution of a metal complex and ligand using a mixed stationary phase containing co-poly(styrene-divinylbenzene) and poly(*N*,*N*,*N*-trimethylammonium-methylstyrene-divinylbenzene) (PRP-X100, Hamilton Co.). The ligand used in this study was 2,6-pyridinediylbis(methylenenitrilo)tetraacetic acid (PBMNTA) complexed with gadolinium (III) to form Gd (III) 2,6-pyridinediylbis(methylenenitrilo)tetraacetic acid (GdPBMNTA) (Fig. 1).

Materials and Methods

Materials

The ligand, 2,6-pyridinediylbis(methylenenitrilo)tetraacetic acid, and the metal complex, the *N*-methylglucamine salt of the gadolinium

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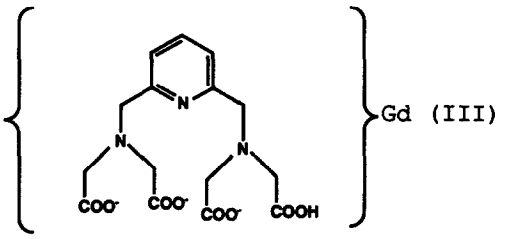

Figure 1
Chemical structure of GdPBMNTA.

(III) complex of 2,6-pyridinediylbis(methylenenitrilo)tetraacetic acid, were provided by Sterling Winthrop Pharmaceuticals Research Division (Rensselaer, NY, USA). Potassium phosphate, dibasic, potassium chloride and sodium chloride were obtained from J.T. Baker (Phillipsburg, NJ, USA); potassium phosphate monobasic, and sodium hydroxide (1.0 N) were obtained from Fisher Scientific (Fair Lawn, NJ, USA); ethylenediaminetetraacetic acid, tetrasodium salt dihydrate was obtained from Aldrich (Milwaukee, WS, USA); citric acid monohydrate, hydrochloride acid (37%), potassium hydroxide were obtained from Mallinckrodt (Paris, KY, USA); ammonium hydroxide (28%) was obtained from Corco Chemical Co. (Fairless Hills, PA, USA). These chemicals were either USP, NF or ACS quality and were used without further purification.

Apparatus

The HPLC system consisted of a Waters 510 solvent delivery system (Waters Associates, Milford, MA, USA), equipped with a single wavelength detector (Spectroflow 757 Absorbance detector, ABI Analytical, Ramsey, NJ, USA), a Waters Maxima 820 data module and a Waters 712 Wisp.

Chromatographic conditions

Samples were separated on a 10 μm, PRP-X100 stainless steel anion-exchange column (Hamilton Co.), 150 × 4.1 mm with an exchange capacity of 0.17 meq g^{-1}. This column was selected because the stationary phase, unlike silica base anion exchangers, is stable up to a pH of 13 [6]. The flow rate was 2.0 ml min^{-1}. The injection volume was 15 μl. Detection was at 260 nm. Standard solutions were prepared in 0.01 N sodium hydroxide. The mobile phases were filtered through a 0.45 micron Nylon filter membrane (Nylon 66, MSI). The following mobile phases were prepared:

To determine the effect of chloride ion concentration on the elution of PBMNTA and GdPBMNTA, a stock solution was prepared containing 0.1 M potassium phosphate, dibasic and 1 mM EDTA, adjusted to a pH of 9.6 by the addition of 0.1 M potassium hydroxide containing 1 mM EDTA. From this stock solution, six different mobile phases were prepared containing potassium chloride at concentrations of 0, 0.025, 0.05, 0.094, 0.1 and 0.2 M KCl. The pH values of these prepared mobile phases were 9.6 ± 0.1.

To determine the effect of pH on the elution of PBMNTA and GdPBMNTA, two stock solutions were prepared keeping ionic strength constant ($\mu = 0.36$) by the addition of potassium chloride. The EDTA concentration was kept constant at 1 mM. The first contained 0.1 M potassium phosphate, dibasic, 0.05 M KCl and 1 mM EDTA. The second stock solution was prepared containing 0.1 M potassium phosphate, monobasic, 0.25 M potassium chloride and 1 mM EDTA. Four different mobile phases at pH values of 7.4, 8.4, 8.9 and 9.4 were prepared by combining the two stocks solutions together to achieve the appropriate pH.

To determine the effect of EDTA concentration on the elution of PBMNTA and GdPBMNTA, six different mobile phases were prepared containing 0.1 M potassium phosphate, dibasic, 0.05 M potassium chloride, and varying concentrations of EDTA (0, 0.05, 0.1, 0.5, 1.0 and 10 mM maintained at constant ionic strength of 0.45 by the addition of sodium chloride). The pH of the mobile phase containing 10 mM of EDTA was adjusted to a pH of 9.2 by the addition of concentrated hydrochloric acid. The pH values of these prepared mobile phases prepared were 9.1 ± 0.2.

To determine the effect of citric acid on the elution of PBMNTA and GdPBMNTA, a mobile phase was prepared containing 0.1 M potassium phosphate dibasic, 0.05 M potassium chloride, 0.1 M sodium chloride and 10 mM of citric acid adjusted to a pH of 9.3 by the addition of concentrated ammonium hydroxide.

Results and Discussion

The effect of chloride ion, pH and EDTA

concentration on the elution of PBMNTA and GdPBMNTA are shown in Tables 1–3 (concentration of each analyte injected onto the column was 500 μg ml^{-1}). The results show that chloride ion concentration, pH of the mobile phase and EDTA effect the elution of PBMNTA and GdPBMNTA. To understand the effect of chloride ion concentration, pH and EDTA concentration on the elution of ligand and chelate, it is necessary to understand the chemistry of the stationary phase, the free ligand and the metal complex. The PRP-X100 is a strong base anion exchanger containing *N,N,N*-trimethyl-ammonium methylstyrene-divinylbenzene (0.2 meq g^{-1}). Approximately one in every seven phenyl 'sites' is functionalized. This stationary phase has been shown to function as an ion exchange phase for organic anions, while retaining some of the original reversed-phase characteristics [6]. PBMNTA forms strong complexes with a large number of metal ions [7], always reacting with metal ions in a 1:1 molar ratio. PBMNTA can be a hexadentate ligand, forming six coordinate bonds through its four carboxylate groups and two nitrilo groups. Increasing the chloride ion concentration of the mobile phase by the addition of potassium chloride decreased the retention time, peak width and resolution of PBMNTA and GdPBMNTA.

Table 1
The effect of chloride ion concentration on the elution of GdPBMNTA and PBMNTA at a constant pH of 9.6 with an EDTA concentration of 1.0 mM

	GdPBMNTA			PBMNTA			
Chloride ion conc. (M)	Retention time (min)	Capacity factor (k)	Peak width (min)	Retention time (min)	Capacity factor (k)	Peak width (min)	Resolution
0.000	11.6	18.3	3.0	43.8	72.0	9.1	5.3
0.025	8.3	12.8	2.1	24.1	39.2	4.6	4.7
0.050	6.8	10.3	1.5	16.3	26.2	3.0	4.2
0.094	5.1	7.5	1.2	9.6	15.0	1.9	2.9
0.100	4.9	7.2	1.1	9.0	14.0	1.7	2.9
0.200	3.4	4.7	0.7	4.3	6.2	1.0	1.1

Table 2
The effect of pH on the elution of GdPBMNTA and PBMNTA at constant ionic strength with an EDTA concentration of 1.0 mM

	GdPBMNTA			PBMNTA			
Mobile phase pH	Retention time (min)	Capacity factor (k)	Peak width (min)	Retention time (min)	Capacity factor (k)	Peak width (min)	Resolution
9.4	6.8	10.3	1.6	17.0	27.3	3.4	4.1
8.9	7.1	10.8	1.7	15.3	24.5	3.6	3.1
8.4	7.5	11.6	1.9	7.6	11.7	3.5	0.0
7.4	6.9	10.5	1.8	1.9	2.2	0.7	4.0

Table 3
The effect of EDTA concentration on the elution of GdPBMNTA and PBMNTA at a constant ionic strength and pH of 9.1

	GdPBMNTA			PBMNTA			
EDTA conc. (mM)	Retention time (min)	Capacity factor (k)	Peak width (min)	Retention time (min)	Capacity factor (k)	Peak width (min)	Resolution
0.00	4.2	6.0	1.3	25.6	41.7	11.1	3.5
0.05	4.2	6.0	1.1	14.9	23.8	11.4	1.7
0.10	4.1	5.9	1.0	10.2	16.0	4.1	2.4
0.50	4.0	5.7	0.9	9.1	14.2	2.1	3.4
1.00	4.1	5.8	0.9	9.6	15.0	2.0	3.8
10.00	5.5	8.2	1.3	12.0	19.0	2.6	3.3

Chloride ion acts as a counter-ion to the quarternary ammonium portion of the stationary phase. This observed effect was in accordance to theoretical considerations [8]. The ionized species, GdPBMNTA and PBMNTA, are retained by displacing the chloride ion. This effect was more noticeable with the free ligand than with the complex. For example, the capacity factor of the ligand decreased from 72 to 6.2 compared to a decrease of 18.3 to 4.7 for GdPBMNTA when the chloride ion concentration was increased from 0 to 0.2 M.

A decrease in mobile phase pH from 9.4 to 7.4, at a constant EDTA concentration of 1.0 mM, decreased the observed retention of the ligand from 17 to 1.9 min but had a lesser effect on changing the retention time of the metal complex with retention times varying from 6.8 to 7.5 min. The ligand, according to the literature, has four pK_a values: 1.5, 3.2, 8.3 and 9.0. The pK_a values at 1.5 and 3.2 correspond to the dissociation of protons on the carboxylic acid groups with the latter corresponding to the dissociation of protons on the two nitrilo groups. At a pH of 7.4, these nitrilo groups are protonated, and therefore should be unable to ion-pair to the positive charge on the stationary phase. Hence the retention of PBMNTA is most likely due to the lone pair of electrons on the two nitrilo groups, rather than the ionized carboxylic acid groups, which formed an ion-pair to the quarternary ammonium portion of the stationary phase.

EDTA was necessary to elute PBMNTA off of the column. The effect of EDTA concentration is shown in Fig. 2. Increasing the concentration of EDTA from 0 to 0.5 mM caused a corresponding decrease in retention time of PBMNTA but did little to change the retention time of GdPBMNTA. Increasing the amount of EDTA from 0.5 to 10 mM actually caused the retention time of the two analytes to slightly increase. This observation was perhaps due to a chloride-ion effect from the sodium chloride used to maintain a constant ionic strength of the mobile phase. The mobile phase containing 10 mM of EDTA had no added sodium chloride. When no EDTA was present in the mobile phase, PBMNTA had a capacity factor of 41.7 producing an asymmetric peak at about 25.6 min. The ideal concentration of EDTA to elute PBMNTA with ideal peak shape was 1 mM yielding a retention time of 9.6 min. EDTA, being a Lewis base, acts to ion-pair to the positive

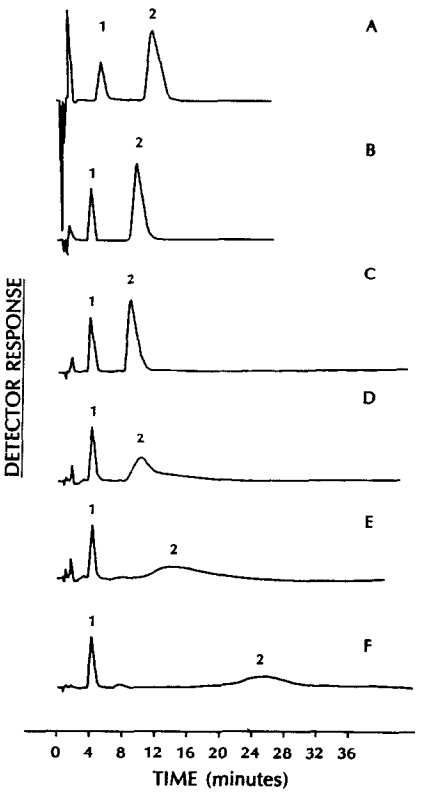

Figure 2
The effect of EDTA concentration on the elution of GdPBMNTA and PBMNTA where A is 10 mM, B is 1.0 mM, C is 0.5 mM, D is 0.1 mM, E is 0.05 mM and F is 0 mM of EDTA. 1, GdPBMNTA; 2, PBMNTA.

charge on the quarternary ammonium portion of the stationary phase; competing with PBMNTA for this site. To determine which functionality of the EDTA molecule, the carboxylic acid group or the nitrilo group, formed an ion-pair to the quarternary ammonium portion of the stationary phase, a mobile phase consisting of 10 mM of citric acid was prepared excluding EDTA. The hypothesis being that if the ionized carboxylic acid groups of EDTA were competitively binding to the positive charge of the stationary phase, then citric acid with its three carboxylic acid groups should exhibit a similar effect to that of EDTA. When a PBMNTA standard (500 μg ml^{-1}) was injected onto the column, PBMNTA eluted at a retention time of 22 min as an asymmetrical peak producing a similar chromatogram to that of a mobile phase containing no EDTA. This suggests that it was the lone pair of electrons on the two nitrilo groups of the EDTA molecule which acted to ion-pair to the positive charge on the quarternary ammonium portion of the stationary phase.

A linear calibration plot was generated for PBMNTA and GdPBMNTA in a mobile phase consisting of 0.1 M potassium phosphate, dibasic, 0.1 M potassium chloride and 1 mM of EDTA. Five standards of PBMNTA were prepared at concentrations ranging from 5 to 80 μg ml^{-1}. For GdPBMNTA, six standards were prepared at concentrations ranging from 20 to 496 μg ml^{-1}. A linear regression analysis using a least square fit was performed by plotting detector response (peak area) on the x-axis against concentration of PBMNTA on the y-axis. The regression output was calculated to be:

$$y = 2.65 \times 10^{-4} x - 0.219, \quad (1)$$

with a correlation coefficient square (r^2) or 0.9996. For GdPBMNTA, the regression output was calculated to be

$$y = 8.95 \times 10^{-4} x + 3.37, \quad (2)$$

with a correlation coefficient square (r^2) of 0.9980. Because EDTA is a strong sequestering agent of metal ions, it was necessary to determine if the EDTA in the mobile phase at 1 mM was a stronger chelator for gadolinium than PBMNTA. Therefore, the same GdPBMNTA standards prepared above were injected onto the column with a mobile phase consisting of 0.1 M potassium phosphate, dibasic and 0.1 M potassium chloride but excluding EDTA. The regression equation obtained was

$$y = 9.09 \times 10^{-4} x + 2.99,$$

with a correlation coefficient square (r^2) of 0.9994, which was similar to the equation derived for GdPBMNTA with EDTA in the mobile phase. Hence, in this instance EDTA had no measureable effect on GdPBMNTA.

Conclusions

An HPLC method was developed which separated PBMNTA from the metal complex GdPBMNTA. The optimal conditions for separating the analytes were best achieved with a mobile phase containing 0.1 M potassium phosphate, dibasic, 0.1 M potassium chloride and 1 mM of EDTA. When using this method for other contrasting agents, the analyst must optimize the counter-ion concentration, EDTA concentration and pH of the mobile phase to obtain the desired resolution. Upon optimization, the dissociation of metal from metal complex can then be monitored.

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