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Note

Isocratic reversed-phase high-performance liquid chromatographic assay for a cryptand–cryptate–free metal ion system

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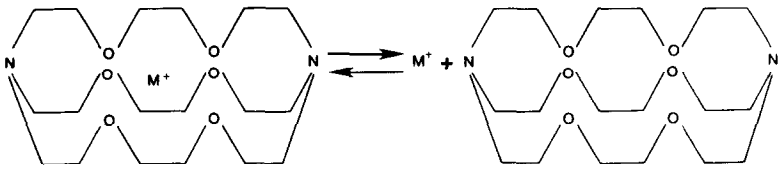
Cryptands are metal ion complexing agents which consist of two bridgehead nitrogens connected by three ethoxy ether bridges. These compounds form inclusion complexes with a variety of metal ions[1]. These complexes, known as cryptates, have varying stabilities dependent upon the relative sizes of the cryptand cavity and the metal ion, the type of solvating medium, and other factors[2–4]. Some potentially practical applications of cryptate complexes include removal of heavy metal ions from contaminated individuals[5], and use as radionuclide carriers for blood flow measurements[6] and in cancer therapy[7].

Assay systems that have been used to determine the extent of cryptate formation from metal ions and cryptands are nuclear magnetic resonance[8,9], conductivity[3,4,10], and enthalpy and volume measurements[10,11]. Recently developed chromatographic methods for assaying metal ion complexing molecules and their corresponding complexes were examined. A number of biogenic amines and some metal complexing species in this category have been separated by acidic solutions or buffers[12,13]. These methods are not acceptable for our purposes since our principal objective was to evaluate the stability of cryptate complexes with a mobile phase which would not promote the equilibrium shown.



Cryptate complexes are known to decompose into the component species at acidic pH as a result of protonation of the bridgehead nitrogens[8]. The free electron pairs on the nitrogens are then unavailable for coordination with the encapsulated metal ion and the complex decomposes into cryptand and free metal ion. Alternatively, Bauer's method for the assay of acidic porphyrins using tetra-N-butylammonium hydroxide solution[14] increased retention times of the components in our system significantly. Therefore, an organic mobile phase appeared most suitable for separation

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of the organic components, *i.e.*, free cryptand and cryptate, followed by an aqueous phase of low pH to remove free metal ions[15]. In view of the potential utility of cryptands in clinical nuclear medicine, we report the development of a high-performance liquid chromatographic (HPLC) assay method for the individual components of a cryptand complexing system.

EXPERIMENTAL

The chromatographic system consisted of a Beckman Instruments (Fullerton, CA, U.S.A.) Model 116 pump, Model 166 UV detector, Model 170 radioisotope detector and Model 210A injector fitted with a 250-μl loop. Detector outputs were monitored by a dual pen strip chart recorder. The column was a Hamilton (Las Vegas, NV, U.S.A.) PRP-1, 150 × 4.1 mm I.D., of 5 μm particle size. Barium chloride (^{133}Ba) was obtained from New England Nuclear (North Billerica, MA, U.S.A.). The cryptand, 4,7,13,16,21,24-hexaoxa-5,6-benzo-1,10-diazabicyclo[8.8.8]hexacosane (2B:2:2), was obtained from PCR (Gainesville, FL, U.S.A.) as a 50% toluene solution. Solvents used were HPLC-grade methanol and water that was purified by Milli-Q Plus system (Millipore, Bedford, MA, U.S.A.). The buffer used to elute free barium ions was prepared from reagent-grade chemicals and consisted of 14.1 m*M* citric acid, 5.88 m*M* sodium dihydrogen phosphate and 0.079 m*M* ethylenediamine tetraacetic acid (EDTA). The buffer was prepared at 10 fold concentration with 20% ethanol by volume. The buffer was diluted as needed and had a pH of 2.7. All solvents were filtered through a sintered glass filter prior to use. The column was protected by a PRP-1 pre-column. Standard methanol solutions were prepared for ultraviolet (UV) analysis from weighed samples of 2B:2:2 recovered from the toluene solution. Spectra were obtained on a Beckman DU-40 ultraviolet–visible spectrometer.

Complex formation

A weighed sample of 2B:2:2 was dissolved in 2 ml of HPLC-graded methanol. In a test tube 5–10 μCi ^{133}BA (20–40 μl) was placed with a slight molar excess of cold $BaCl_2$ and evaporated to dryness. The methanol solution of 2B:2:2 was added to the $BaCl_2$ residue and the resulting mixture was mixed for 5 min. The solution was allowed to stand for 15 min. The methanol was then evaporated under a stream of nitrogen and the oily residue was extracted two times with 1 ml of methylene chloride. The extracts were dried over anhydrous sodium sulfate, decanted, and taken to dryness under a stream of nitrogen. The residue was dissolved in 2 ml of HPLC-grade methanol and filtered through a 0.45-μm filter prior to analysis.

Method of chromatography

Methanol was used to elute 2B:2:2 and its corresponding complex with Ba^{2+}. The column was then washed with the citrate–phosphate buffer to elute free barium ions. Sample size varied from 10–100 μl. The flow-rate was 0.5 ml/min. The system was recycled between runs by washing the column with methanol–water (2:1).

HPLC column recoveries

Each component of the mixture in the complex equilibrium was subject to column recovery experiments using either the UV absorbance at 275 nm (2B:2:2) or

scintillation counting of radioactivity (complex and free Ba^{2+}). Counting standards and a standard curve generated from the UV absorbance of known concentrations of 2B:2:2 at 275 nm were used to determine percent recoveries of peaks eluted from the column. Scintillation counting of samples was performed on a Beckman gamma 4000.

RESULTS AND DISCUSSION

Both 2B:2:2 and the 2B:2:2 complex with barium ion were eluted from the column in methanol, with retention volumes of 5.5 ml and 1.5 ml respectively. The buffer (citric acid–dihydrogenphosphate–EDTA) quickly and efficiently removed barium ions, presumably as complexed salts. Barium was eluted with a retention volume of 7.0 ml once the solvent change was effected. Interestingly, free barium was not eluted from the column using methanol as a mobile phase. This result does not indicate any particular affinity for the stationary phase, but more likely reflects the reduced solubility of barium salts in methanol[16]. In addition, separation of the components of the equilibrium system using methanol, which is a weaker acid than water, indicates that the establishment of chemical equilibrium in methanol is slow, relative to the time required for the analysis. Cryptate complexes are known to be considerably more stable in methanol than in water[1]. A typical elution pattern of a mixture containing all three components of the system under study is provided in Fig. 1. The results of the column recovery experiments are provided in Table I. Ultraviolet spectra of 2B:2:2 showed that absorbance at 275 nm was linear with concentration of 2B:2:2 over the range studied.

Our interest in the potential use of cryptate complexes of metal radionuclides for cancer therapy required the development of a convenient assay procedure for

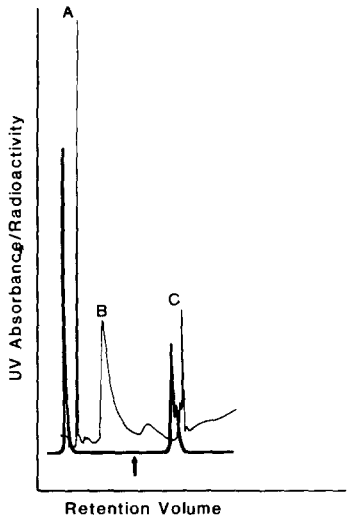

Fig. 1. Typical elution pattern of an equilibrium mixture of barium complex of 2B:2:2 (A), 2B:2:2 (B) and uncomplexed barium (c) represented by UV absorbance (———), and radioactivity (———). Arrow indicates point of mobile phase change.

TABLE I

HPLC COLUMN RECOVERIES (%)

Species	*Trials*	*Recovery (%)*
2B:2:2	6	87.5[a] ± 1.53
Complex	8	101.8[b,c] ± 1.91
Ba^{2+}	5	92.4[c] ± 3.94

[a] By UV absorbance at 275 nm.
[b] Recovery consists of 74.9 ± 6.17% complex and 26.9 ± 5.67% excess free Ba^{2+}.
[c] By scintillation counting of ^{133}Ba.

determination of complex stabilities under various conditions. The method reported here is the first chromatographic assay developed for this system and is more convenient than the assay methods previously reported.

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