Chromatographic Methods for the Quantification of Free and Chelated Gadolinium Species in MRI Contrast Agent Formulations

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Abstract

Speciation measurements of gadolinium in liposomal MRI contrast agents are complicated by the presence of emulsifiers, surfactants, and therapeutic agents in the formulations. The present paper describes two robust, hyphenated chromatography methods for the separation and quantification of gadolinium in nanoemulsion-based contrast agent formulations. Three potential species of gadolinium, free gadolinium ion, gadolinium chelated by diethylenetriamine pentaacetic acid (Gd-DTPA), and gadolinium chelated by diethylenetriamine pentaacetic acid-phosphatidylethanolamine (Gd-DTPA-PE), were present in the contrast agent formulations. The species were separated by reversed-phase chromatography

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(RP-HPLC), or by high pressure size exclusion chromatography (HPSEC). For RP-HPLC, fluorescence detection and post-column, online isotope dilution inductively coupled plasma mass spectrometry (ID-ICP-MS) were used to measure the amount of gadolinium in each species. Online ID-ICP-MS and triple-spike species-specific isotope dilution (SID) ICP-MS were used in combination with the HPSEC column. The results indicated that some inter-species conversions and degradation had occurred within the samples, and that SID-ICP-MS should be used to provide the most reliable measurements of total and speciated gadolinium. However, fluorescence and online ID-ICP-MS might usefully be applied as qualitative, rapid screening procedures for the presence of free gadolinium ions.

Keywords: gadolinium speciation, MRI contrast agent formulations, isotope dilution analysis, species-specific isotope dilution analysis, ICP-MS, fluorescence detection, high pressure size exclusion chromatography, reversed-phase chromatography, nanoemulsion, DTPA-PE, liposome

Introduction

Magnetic resonance imaging (MRI) is a prominent clinical diagnostic tool in the health-care community for *in vivo*, noninvasive tissue characterization. Briefly, the MRI technique is based on the spatial detection of the relaxation of water protons in the tissue of interest following application of an external magnetic field. The external magnetic field induces a net magnetic field between the two protons in each water molecule.[1] The net magnetization is first perturbed by application of a radio frequency (RF) pulse, and then allowed to relax to its equilibrium value.[2] An MRI image is acquired by rapid alternation between perturbation and relaxation of the net magnetic field *via* repeated application of RF pulses. Structural information about the

tissue of interest is conveyed as a function of the variation of the signal level across the image, which is dependent on the density of the water protons, and on the relaxation time of the net magnetization.[3] Unfortunately, for cases where neighboring tissues have the same relaxation times, the differences in signal levels can become too low, rendering the tissues indistinguishable.[4]

In these cases, paramagnetic atom-based contrast agents can be applied to alter the relaxation times of the tissues of interest. The unpaired electrons in a paramagnetic atom have electron spin, which inherently creates a stronger net magnetic field than that created by the water protons. In this way, the degree of perturbation induced by the applied RF pulses is increased, the relaxation times are shortened, and the differences in signal levels among adjacent tissues are enhanced. The gadolinium ion, which has seven unpaired electrons, has been widely applied as an MRI contrast agent by the clinical community. Although the free gadolinium ion has potentially toxic effects *in vivo*, complexation of the ion by various ligands has been shown to improve acute tolerance. Several contrast agent formulations employing chelated gadolinium have been approved by the U.S. Food and Drug Administration. For example, the commercial formulation Magnevist (gadopentetate dimeglumine), first approved for use in 1988, contains gadolinium chelated by diethylenetriamine pentaacetic acid (DTPA).

One existing challenge during the formulation and application of novel MRI contrast agents is the possibility of the presence of uncomplexed, or free, gadolinium ions, which may be due to events such as incomplete gadolinium-ligand complexation, release of the metal ion from the ligand over time, and *in vivo* transmetallation.[5,6] As previously mentioned, the gadolinium ion is toxic in the body, and has been known to precipitate *in vivo* with inorganic phosphate and carbonate, and to have a significantly slower rate of excretion compared to chelated forms.[7,8]

Therefore, it is crucial to develop methods that accurately quantify the amounts of both complexed and uncomplexed gadolinium species in contrast agent formulations. Ideally, these methods should be robust and use equipment routinely found in the clinical setting.

In the literature, several different approaches have been developed for the determination of total and speciated gadolinium in MRI contrast agent formulations and other media. These methods have included online cloud point extraction coupled with spectrophotometric detection[9] and quantification with inductively coupled plasma optical emission spectrometry (ICP-OES),[10] and colorimetric testing using the Arsenazo III reaction.[11,12] Liquid chromatographic separations have also been applied for the simultaneous determination of free and complexed gadolinium species. Various types of stationary phases, including reversed-phase,[13,14,15,16] ion exchange,[17,18] ion pair,[19] hydrophilic interaction,[20] and size exclusion columns,[21] have been explored in the literature for gadolinium speciation. For example, Tweedle and co-workers[14,15] devised two C18 reversed-phase separation methods (RP-HPLC) with fluorescence detection for the determination of free gadolinium ions in gadolinium-tetraazacyclododecanetetraacetic (Gd-DOTA) and Gd-DTPA. The ligands ethylenediamenetetraacetic acid (EDTA) and 1,2-cyclohexylenedinitrilotetracetic acid (CDTA) were added to the mobile phases to complex any free gadolinium ions in the samples as the mobile phase swept the sample out of the sample loop, with no sample preparation requirements. This inline chelation scheme created the Gd-EDTA or Gd-CDTA species, respectively, which allowed for the use of fluorescence detection, and was intended to introduce a lipophilic component to the gadolinium ion so that retention and separation could be achieved on the reversed-phase columns. The authors noted little to no separation of Gd-EDTA, Gd-DTPA, and Gd-DOTA, but were able to successfully separate Gd-DTPA and Gd-CDTA. Similarly, Moutiez *et al.*[16] coupled RP-HPLC on a C8 column with time

resolved luminescence detection to quantify free gadolinium ions in the contrast agent Gd-DOTA. The authors used CDTA to chelate any free gadolinium that was in the sample. The Gd-DOTA species eluted at approximately 3 min, while Gd-CDTA eluted at around 6 min.

Loreti and Bettmer[21] coupled high pressure size exclusion chromatography (HPSEC) to inductively coupled plasma mass spectrometry (ICP-MS) to simultaneously determine free and bound gadolinium in Gd-DTPA standards, and in urine, hair, saliva, and sweat samples obtained from a patient that had been intravenously administered Magnevist. No gadolinium species were detected in the saliva and sweat samples, and only the Gd-DTPA species was detected in urine. Both Gd-DTPA and free gadolinium ions were observed in hair samples, but it was determined that the digestion method that was used for the hair samples was responsible for the release of the free gadolinium ions from the Gd-DTPA, and that no free gadolinium ions were present in the native hair samples.

The present paper describes two hyphenated liquid chromatography methods for the simultaneous measurement of free and complexed gadolinium species in the presence of emulsifiers, surfactants, biomarkers, and oils in nanoemulsion-based MRI contrast agent formulations. In these formulations, the liposomal chelator diethylenetriamine pentaacetic acid-phosphatidylethanolamine (DTPA-PE) was used as the chelating agent for gadolinium, and the resultant species has been termed Gd-DTPA-PE in this work. These types of paramagnetic liposomes have shown promise as highly potent contrast agents, due to their accommodation of a significantly higher gadolinium payload, improved relaxivities, and reduced incidence of release of gadolinium ions compared to traditional contrast agents.[22,23] The first method described here coupled a reversed-phase C12 chromatography column with fluorescence (FL) detection. The chelator EDTA was added to the aqueous mobile phase to bind any free gadolinium in the

samples directly in the sample loop. Additionally, the output from the fluorescence detector was coupled to an ICP-MS for analysis by post-column isotope dilution mass spectrometry (ID-ICP-MS),[24] which is referred to as online ID-ICP-MS in the present paper. A second method used HPSEC coupled to online ID-ICP-MS to quantify free, bound, and total gadolinium in the samples. Finally, multiple-spike species-specific isotope dilution (SID)[25] was used in combination with HPSEC-ICP-MS to quantify free and bound gadolinium species while simultaneously compensating for on-column losses and inter-species transformations. The experimental results for RP-HPLC-FL were then compared to the results for RP-HPLC-ID-ICP-MS, HPSEC-ID-ICP-MS, and HPSEC-SID-ICP-MS, as well as to the results for the ID-ICP-MS analysis of total gadolinium in the nanoemulsions.

Experimental[1]

Samples and reagents. Deionized water was prepared in house with a specific resistance of 18 MΩ-cm. Standard solutions of each chelated gadolinium species were prepared in the lab by dissolution of the chelating agents EDTA (Fisher Scientific, Pittsburgh, PA), DTPA (Sigma Aldrich, St. Louis, MO), and DTPA-PE (Avanti Polar Lipids, Alabaster, AL) in a laboratory-prepared 50 mM Tris (GFS Chemicals, Inc., Powell, OH) buffer solution (pH 7.4). An equimolar amount of gadolinium was then added to the chelator solutions, and the mixtures were adjusted to pH 7.4 to stabilize the complexes and prevent ligand substitution.[13] For natural

1. Certain commercial equipment, instruments, or materials are identified in this paper to foster understanding. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

gadolinium-ligand standard solutions, denoted in the present work as ^{nat}Gd-DTPA-PE, ^{nat}Gd-DTPA, and ^{nat}Gd-EDTA, the gadolinium source was NIST SRM 3118a. For species-specific isotope dilution analysis, isotopically-enriched gadolinium-ligand standard solutions, denoted in the present work as ^{155}Gd-DTPA-PE, ^{156}Gd-DTPA, and ^{160}Gd-EDTA, were prepared using enriched gadolinium isotopes that had known isotopic compositions. The ^{155}Gd, which was obtained from Oak Ridge National Laboratory (Oak Ridge, TN), had an isotopic enrichment of 99.82 %, while the ^{156}Gd and ^{160}Gd were obtained from Trace Sciences International (Richmond Hill, ON) and had isotopic enrichments of 95.50 % and 98.40 %, respectively. The amounts of gadolinium in the ^{155}Gd-DTPA-PE, ^{156}Gd-DTPA, and ^{160}Gd-EDTA enriched species solutions were measured by reverse isotope dilution analysis with NIST SRM 3118a. Measurements of total gadolinium by ID-ICP-MS and speciated gadolinium by online ID-ICP-MS were performed using a lab-prepared ^{157}Gd enriched spike solution that had an isotopic enrichment of 88.63 % (Oak Ridge National Laboratory, Oak Ridge, TN). Lipoid E-80 was obtained from Lipoid GMBH (Ludwigshafen, Germany), and sodium dodecyl sulfate (SDS) was obtained from Polysciences, Inc. (Warrington, PA). The nanoemulsion samples were provided by Professor Mansoor Amiji's laboratory at Northeastern University (Boston, MA) through the National Cancer Institute's Nanotechnology Characterization Laboratory (NCL) (Frederick, MD), and the composition of each nanoemulsion is given in Table 1. Details of the preparation of the nanoemulsions have been described elsewhere.[26,27,28] For this work, the nanoemulsions were mixed well with a mini vortexer prior to and during use, and stored in a refrigerator at approximately 4 °C between uses.

Total gadolinium measurements by isotope dilution inductively coupled plasma mass spectrometry. The total, non-speciated amount of gadolinium in each nanoemulsion

sample was determined by isotope dilution ICP-MS. In this work, a known amount of a known concentration of a ^{157}Gd enriched isotope spike solution was added to each sample, and the mixtures were then diluted to appropriate concentrations for ICP-MS analysis in 2 % nitric acid or a mixture of 3 % hydrochloric + 1 % nitric acids. The target ratio range for the $^{157}Gd/^{158}Gd$ measurements was 3 – 4 in order to minimize error magnification. A standard flow glass concentric nebulizer was used to introduce samples into a water-cooled (4 °C) low volume cyclonic spray chamber. The gadolinium isotope ratio $^{157}Gd/^{158}Gd$ was measured using a quadrupole-based ICP-MS (PlasmaQuad 3, VG Elemental, Winsford, Cheshire, UK), with a dwell time of 10 ms for all masses. The ICP-MS was operated at 1350 W with standard gas flows, and equipped with a platinum-tipped sampler cone and a nickel skimmer cone. The measured ratios were corrected for mass discrimination, and the concentration of the enriched ^{157}Gd isotope in the spike solution was determined by reverse isotope dilution analysis. Masses ^{137}Ba, ^{139}La, ^{140}Ce, ^{141}Pr, ^{146}Nd, and ^{163}Dy were monitored for potential interference from oxides and hydroxides of these elements on the gadolinium masses of interest, but none were present in any significant amount.

Chromatographic methods for gadolinium speciation and quantification. A variety of analytical stationary phases was investigated during the method development stage. These phases included C18, C12, C8, silica, cyano, amino, cation and anion exchange, and size exclusion. For all phases, the separations were evaluated under strong solvent elution conditions, aqueous elution conditions, and low pH conditions. The separations of bound and free gadolinium species were best achieved using the C12 and size exclusion columns. The optimized analysis conditions for each of these are summarized in Table 2, and the resulting analytical methods are described in depth in the following sections.

Reversed-phase chromatography with fluorescence detection. For reversed-phase chromatographic separations, a Synergi Max-RP C12 column (Phenomenex, Inc., Torrance, CA; 250 mm x 4.6 mm id, 4 μm particle size, TMS endcapped) was used at ambient temperature. A Varian 9012 HPLC pump (Varian, Walnut Creek, CA) was used to deliver an isocratic mobile phase, comprised of 50 mM Tris + 2 mM EDTA that had been adjusted to pH 7.4, at a flow rate of 1 mL min^{-1}. The chelating agent EDTA was added to the mobile phase to bind any free gadolinium ions in the samples directly in the sample loop, which resulted in the formation of the Gd-EDTA species. In this way, no sample preparation or pretreatment steps were required prior to sample analysis. The other species of interest, Gd-DTPA and Gd-DTPA-PE, in the samples were stable at neutral pH and were not affected by the presence of excess EDTA. Also, the chelated gadolinium species Gd-EDTA and Gd-DTPA fluoresced under the Tris conditions used for the reversed-phase method. This method is similar to the approach described by Tweedle and co-workers,[14,15] and was based on the separation of components by their relative hydrophobicities. In other words, under 100 % aqueous conditions, the more hydrophobic a component was, the longer it was retained on the reversed-phase column. Samples and standards were injected onto the column using a manual injector (Rheodyne, model 7125, Cotati, CA) equipped with a 10 microliter sample loop. Polyetheretherketone (PEEK) tubing was used to make all connections in the experimental arrangement. In order to avoid the phase dewetting and retention loss phenomena associated with using all-aqueous mobile phases on reversed-phase columns, conditioning and re-equilibration steps were used between analyses. The conditioning step consisted of rinsing the column with 100 % HPLC-grade methanol (Mallinckrodt Baker, Inc., Phillipsburg, NJ) for 5 min. The column was then re-equilibrated with the mobile phase for 15 min. Fluorescence spectra were collected on a fluorescence detector (Jasco FP-1520

Intelligent Fluorescence Detector, Jasco, Inc., Easton, MD) with an excitation wavelength of 280 nm and an emission wavelength of 316 nm. The fluorescence data were collected using an analog-to-digital converter (Advanced Computer Interface, Dionex Corporation, Sunnyvale, CA) and Dionex AI-450 software (Dionex Corporation, Sunnyvale, CA), and were processed using spreadsheet software (Microsoft Excel 2003, Microsoft Corp., Redmond, WA). The amounts of gadolinium in the unknown samples were quantified by injecting lab-prepared aqueous ^{nat}Gd-EDTA standards with known gadolinium concentrations onto the column. The fluorescence detector gave a linear, concentration-dependent response, so in this way, the areas of the fluorescence signals of the standards were used to create a calibration graph for quantification of the gadolinium in the nanoemulsion samples.

Online ID-ICP-MS was used to assess the results obtained by the fluorescence detection method. The eluent from the fluorescence detector was coupled directly into a stainless steel tee connector, where it was mixed with a known concentration of ^{157}Gd enriched isotopic spike solution. The spike solution was pumped into a stainless steel tee with a peristaltic pump, and had a constant flow rate of 0.49 mL min^{-1}. The resulting eluent-spike mixture was then pumped into the ICP-MS for measurement of the ^{157}Gd/^{158}Gd ratio as a function of time. The data analysis method for the RP-HPLC-ID-ICP-MS measurements was based on the procedure devised by Rottmann and Heumann.[24] The overall experimental arrangement for chromatographic separations coupled to fluorescence detection and online ID-ICP-MS detection is shown in Figure 1A.

High pressure size exclusion chromatography with online isotope dilution inductively coupled plasma mass spectrometry. High pressure size exclusion separations (HPSEC) were carried out at ambient temperature on a BioSep-SEC-S2000 column (Phenomenex, Inc.,

Torrance, CA; 300 x 7.80 mm, 5 μm particle size). The HPSEC column separated sample components by physical size, probably due to differences in hydrodynamic volumes. Characteristically, the largest component (Gd-DTPA-PE) eluted first because it was excluded from many of the pores in the stationary phase, while the smallest component (Gd-EDTA) eluted last due to its ability to enter the pores. A Varian 9012 HPLC pump was used to deliver an isocratic mobile phase, which consisted of a solution of 17 mM SDS + 2 mM EDTA solution that had been adjusted to pH 7. Under these conditions, the exclusion range of the column was approximately 200 – 75,000 Da. It should be noted that significant tailing was observed at the location of the free gadolinium peak when EDTA was omitted from the mobile phase. Since the mass of the free gadolinium ion was below the permeation limit for the column, it was suspected that the tailing resulted from adsorption or other secondary column interactions.[29] Therefore, 2 mM EDTA was added to the mobile phase to simultaneously chelate any free gadolinium in the samples and to reduce adsorption *via* the increase in the mass of the free gadolinium, as Gd-EDTA, above the column permeation limit. The flow rate was 0.7 mL min^{-1}, and the sample injection loop had a volume of 10 microliters. Unfortunately, the gadolinium species did not fluoresce under SDS conditions, so only ICP-MS detection was used to quantify the amount of gadolinium in each species. As before, the eluent from the column was coupled into a stainless steel tee connector, where it was mixed with a ^{157}Gd enriched isotopic spike. The resulting mixture was then pumped into the ICP-MS, which had been equipped with a standard flow glass concentric nebulizer, for temporal measurement of the $^{157}Gd/^{158}Gd$ ratio. Data analysis for the HPSEC-ID-ICP-MS measurements was identical to that used for RP-HPLC-ID-ICP-MS, and based on the procedure devised by Rottmann and Heumann.[24] It should be noted that the

HPSEC column was compatible with 100 % aqueous mobile phases, and no conditioning or re-equilibration steps were required between analyses.

High pressure size exclusion chromatography with triple-spike species-specific isotope dilution inductively coupled plasma mass spectrometry. Triple-spike SID-ICP-MS was also performed on the HPSEC column. As previously described, standard solutions of chelated, isotopically enriched gadolinium species were prepared in the lab. Appropriate amounts of each of the three enriched gadolinium-ligand standards, ^{155}Gd-DTPA-PE, ^{156}Gd-DTPA, and ^{160}Gd-EDTA, were then added to an aliquot of each NCL nanoemulsion. The volumes of the enriched standards were such that the target measured ratios would range from 6 – 8 for ^{155}Gd/^{158}Gd, ^{156}Gd/^{158}Gd, and ^{160}Gd/^{158}Gd. These triple-spiked mixtures were then chromatographically separated using the HPSEC method previously described. As shown in Figure 1B, the eluent was then pumped directly into the ICP-MS for time-dependent measurement of the ratios ^{155}Gd/^{158}Gd, ^{156}Gd/^{158}Gd, and ^{160}Gd/^{158}Gd. The measured ratios were corrected for mass discrimination, and the concentration of gadolinium in each species in the nanoemulsion formulations was calculated using a spreadsheet (Microsoft Excel 2003, Microsoft Corp., Redmond, WA) devised to solve three unknowns in three equations. The species-specific isotope dilution approach is unique because accurate results can be obtained regardless of poor recoveries and on-column losses. The technique can also be used to track inter-species transformations that occur in the sample matrix or during the chromatographic separations.

Results and Discussion

Measurement of total gadolinium by ID-ICP-MS. The results for measurement of the total gadolinium in the nanoemulsion samples by ID-ICP-MS are given in Table 3. These

measurements were made on three different occasions in order to evaluate the stability of the nanoemulsions with respect to the total gadolinium concentration during long-term refrigerated storage (4 °C). As shown in Table 1, no gadolinium or biomarkers were intentionally added to nanoemulsion formulation NCL 55-4. The ID-ICP-MS measurements found no significant amounts of gadolinium in the sample at any time, which indicated that the formulations were not contaminated with gadolinium during the preparation, handling, and storage procedures carried out by the present authors. Rather, the very small amounts of gadolinium measured in NCL 55-4 were probably due to gadolinium contamination in the individual components of the formulation, which were not supplied for testing. Samples NCL 56-3, NCL 57-2, NCL 58-2, and NCL 59-2 were each formulated with 100 mg of Gd-DTPA-PE, and differed only in the amounts and identities of the biomarkers that had been added to the samples. As shown in Table 3, the total amount of gadolinium quantified for these samples appeared to have increased between the analyses performed in late 2007 and those performed in early and mid 2009. The nanoemulsions were refrigerated (4 °C) and stored in snap-top vials, so it is suspected that the gadolinium concentration was increased by the transpiration of water during long-term storage. Unfortunately, it is not clear whether or not the gadolinium species in the samples were equally affected by water loss. A Student's t-test was used to determine whether or not, at a 95 % confidence level, the total measured amount of gadolinium in each sample had statistically changed as a function of time. The comparisons indicated that the results for total gadolinium that were obtained in September 2007 were statistically different than those obtained in July 2009 for all of the nanoemulsions except the blank NCL 55-4 sample. The t-tests also indicated that there were no significant differences, at a 95 % confidence level, between the results of the total gadolinium measurements made in January 2009 and in July 2009. The results for

September 2007 and January 2009 were found to be statistically the same, at 95 % confidence level, for all of the samples except NCL 57-2 and NCL 59-2. The percent differences between the 2007 and 2009 measurements ranged from 7-14 %. It should be noted that all of the expanded uncertainties given in this paper were calculated using the components of uncertainty listed in Table 4.

Gadolinium speciation using reversed-phase chromatography with fluorescence detection and online isotope dilution analysis. Examples of reversed-phase separations on a C12 column with fluorescence detection are shown in Figure 2A for known standard solutions of Gd-EDTA, Gd-DTPA, and Lipoid E-80. The spectra have been offset for clarity. Deionized water was injected as a blank sample, and no fluorescence was observed. The Gd-EDTA species eluted near the void volume, at approximately 3 min, while the Gd-DTPA species eluted at around 5 min. A fluorescence signal was also observed for the emulsifier Lipoid E-80, which eluted at approximately 6 min. The Gd-DTPA-PE species appeared to have been irreversibly retained on the column under the aqueous chromatographic conditions because no fluorescence signal was observed for a Gd-DTPA-PE standard. As described later, ICP-MS detection confirmed the complete retention of the Gd-DTPA-PE species on the C12 column. This inability to achieve mass balance was probably due to the hydrophobicity of the PE moiety, but it should be noted that the Gd-DTPA-PE species also appeared to have remained irreversibly bound to the column despite the use of strong solvents, such as 100 % methanol, 100 % acetonitrile, and 100 % tetrahydrofuran as the mobile phase. In this way, the amount of gadolinium in the Gd-DTPA-PE species could have been determined only by subtraction of the free gadolinium and Gd-DTPA species from the total obtained by ID-ICP-MS analysis.

The fluorescence spectra of the NCL nanoemulsion samples following reversed-phase separations are given in Figure 2B. Sample NCL 55-4 gave only a fluorescence signal at 6 min, corresponding to Lipoid E-80. Sample NCL 56-3 gave a moderate peak at 3 minutes, while nanoemulsion samples NCL 57-2, NCL 58-2, and NCL 59-2, gave small to barely visible peaks at 3 min, indicating that some free gadolinium was present in the formulations. Those samples also contained Lipoid E-80, and a large peak was observed at 6 min for each of the nanoemulsions, corresponding to the elution time of the Lipoid E-80 standard. The NCL nanoemulsions also appeared to give fluorescence signals at around 5 min, which corresponded to the elution time of the Gd-DTPA species. The presence of Gd-DTPA in the samples was probably due to degradation of the Gd-DTPA-PE species as a function of time. Additionally, the Gd-DTPA species could have been formed due to the partial cleavage of the PE moiety from the Gd-DTPA-PE species in the formulations or during separations, or due to an impure DTPA-PE starting material that contained some DTPA. The amounts of free gadolinium, as Gd-EDTA, that were quantified by fluorescence spectroscopy in the nanoemulsions are given in Table 5.

To correlate the presence of gadolinium with fluorescence peaks, the eluent from the fluorescence detector was immediately directed into an ICP-MS for elemental analysis. The ICP-MS results confirmed that only two gadolinium-containing species were eluted from the column. The first species, which was observed at 3 min, corresponded to the elution time of free gadolinium as Gd-EDTA. A second gadolinium-containing species eluted at approximately 5 min, which was consistent with the elution time of Gd-DTPA. No gadolinium was associated with the Lipoid E-80 peak that eluted at 6 min, and no gadolinium signal was observed for the Gd-DTPA-PE species using ICP-MS detection. These results corroborated the FL results that the Gd-DTPA-PE species had been irreversibly bound to the reversed-phase column.

As previously described, online isotope dilution mass spectrometry with a ^{157}Gd enriched isotopic spike solution was performed to measure the amount of gadolinium associated with each eluted peak. An example of the temporal ICP-MS chromatogram for online isotope dilution following the reversed-phase separation of nanoemulsion NCL 56-3 is shown in Figure 3. The ICP-MS was set to measure ion counts as a function of time at masses 157 and 158 for gadolinium. As shown in Figure 3, before the Gd-EDTA species eluted at 3 min, the temporal signal intensities at masses 157 and 158 were due solely to the spike solution, which resulted in the measurement $^{157}Gd/^{158}Gd$ ratio that was higher than the natural $^{157}Gd/^{158}Gd$ ratio. The sample contained the natural isotopic ratio of $^{157}Gd/^{158}Gd$, so when the free gadolinium, as Gd-EDTA, in the sample eluted from the column at 3 min, there was an additional contribution, from the sample, to the signal intensities at both masses 157 and 158 for gadolinium. In other words, when the enriched spike and eluted sample were mixed online in the stainless steel tee, the enriched ratio of $^{157}Gd/^{158}Gd$ in the spike and the natural ratio of $^{157}Gd/^{158}Gd$ in the sample were combined to produce a new $^{157}Gd/^{158}Gd$ ratio that depended on the amount of enriched spike that was added and on the amount of natural gadolinium initially present in the sample. The original ratios in the spike and sample, and the new, combined ratio, are mathematically related by the online isotope dilution mass equation, which has been described in detail elsewhere.[24] In this way, the ratios of masses $^{157}Gd/^{158}Gd$ that were measured as a function of time during the reversed-phase separations were used to calculate the concentration of gadolinium, by species, in the NCL nanoemulsion formulations. The amounts of free gadolinium, as Gd-EDTA, and gadolinium bound as Gd-DTPA that were measured by RP-HPLC-ID-ICP-MS are compared to the results for RP-HPLC-FL in Table 5. A Student's t-test was used to determine whether or not the speciated results were statistically the same for RP-HPLC-FL and RP-HPLC-ID-ICP-MS at a

95 % confidence level. For both the Gd-DTPA and Gd-EDTA species, the results were statistically the same for fluorescence and online ID-ICP-MS detection for all of the samples.

Gadolinium speciation using high pressure size exclusion chromatography with online isotope dilution inductively coupled plasma mass spectrometric detection. As previously mentioned, the Gd-DTPA-PE species appeared to have been irreversibly bound to the C12 column under aqueous and strong solvent conditions, so mass balance could not be achieved using the reversed-phase separation method. Therefore, a high pressure size exclusion chromatography (HPSEC) speciation method was devised to achieve mass balance and to validate the reversed-phase separation results. Because the gadolinium species did not fluoresce under the SDS mobile phase conditions required for size separation, ICP-MS detection was used to quantify the gadolinium in each species as it eluted from the column.

In Figure 4, examples of overlaid HPSEC-ICP-MS chromatograms that were recorded at mass 158 for gadolinium show elution times for standard solutions of ^{nat}Gd-DTPA-PE, ^{nat}Gd-DTPA, and ^{nat}Gd-EDTA, compared to a chromatogram for nanoemulsion sample NCL 59-2. The ^{nat}Gd-EDTA standard, identified by the medium gray signal trace with diamond markings, eluted at 12 min, while the ^{nat}Gd-DTPA standard, identified by the dark gray trace with triangle markings, eluted at 11 min. A ^{nat}Gd-DTPA-PE standard, represented by the light gray trace with square markings, gave a peak at 9 min for the Gd-DTPA-PE species. The HPSEC separation of NCL 59-2, identified by the black trace with circle markings, gave gadolinium peaks at 9, 11, and 12 min, indicating that the nanoemulsion sample contained all three gadolinium species. The relative intensities of the peaks at mass 158 for gadolinium in the nanoemulsion indicated that the majority of the gadolinium in the sample existed as the bound Gd-DTPA-PE species,

which as previously described, is desirable for contrast agent formulations. Less gadolinium was unbound, or free, in the sample, while still less existed as the Gd-DTPA species.

The amounts of gadolinium in each species were quantified using HPSEC coupled with online isotope dilution with a ^{157}Gd enriched isotopic spike, and the results are given in Table 6. The total gadolinium, quantified by HPSEC-ID-ICP-MS and listed in Table 6, was defined in the present work as the sum of the measured amounts of gadolinium in the three species, namely Gd-DTPA-PE, Gd-DTPA and free gadolinium as Gd-EDTA.

A Student's t-test was used to compare the amounts of gadolinium in each species that was measured by RP-HPLC-ID-ICP-MS and HPSEC-ID-ICP-MS. The results for the Gd-DTPA species were all statistically the same, at a 95 % confidence level, for all samples except NCL 55-4. The results for the free gadolinium, measured as Gd-EDTA by RP-HPLC-ID-ICP-MS and HPSEC-ID-ICP-MS, were statistically different for all samples, except NCL 56-3. A Student's t-test was also used to compare speciation measurements by RP-HPLC-FL and HPSEC-ID-ICP-MS. At a 95 % confidence level, the results for both the Gd-DTPA and Gd-EDTA species were all statistically the same. In other words, although there was some agreement in the results among the three quantification schemes, there were also significant discrepancies. Further, comparisons of the measurements of total gadolinium by ID-ICP-MS (Table 3) with the measurements of total gadolinium by HPSEC-ID-ICP-MS (Table 6) indicated a significant on-column loss of gadolinium. It appeared that an average of only 23 % of the total amount of gadolinium in the NCL nanoemulsions was recovered from the HPSEC column. It is unknown whether the losses were equal among all three gadolinium species. Also, some inter-species transformations may have occurred in the formulations, or during the chromatographic separation, that may have impacted the RP-HPLC-FL-ID-ICP-MS and HPSEC-ID-ICP-MS

results in unknown ways. Species-specific isotope dilution analysis was performed to address these concerns.

Gadolinium speciation using high pressure size exclusion chromatography with triple-spike species-specific isotope dilution inductively coupled plasma mass spectrometric detection. The amounts of gadolinium per species that were measured by triple-spike HPSEC-SID-ICP-MS are given in Table 6. As before, the total gadolinium was determined by summation of the measured amounts of gadolinium in the three species. The results indicated that for NCL 56-3, approximately 77 % of the total gadolinium was bound as Gd-DTPA-PE, 16 % was free, and 7 % was bound as Gd-DTPA. The addition of biomarkers paclitaxel and ceramide-C6 appeared to have decreased the amount of free gadolinium in the nanoemulsion formulations. Samples NCL 57-2 and 58-2 each contained approximately 4 % of their total gadolinium as free gadolinium, while NCL 59-2 contained approximately 2 % free gadolinium. The amounts of gadolinium bound as Gd-DTPA-PE and Gd-DTPA, respectively, were approximately 90 % and 6 % for NCL 57-2, NCL 58-2, and NCL 59-2. A Student's t-test was used to compare the measured amounts of gadolinium in each species by HPSEC-ID-ICP-MS and HPSEC-SID-ICP-MS at 95 % confidence level. The results for each species were statistically different for all samples, except the Gd-EDTA species measurements in NCL 55-4, NCL 57-2, and NCL 58-2.

A Student's t-test was also used to compare the results for total gadolinium obtained by ID-ICP-MS in July 2009 with the totals measured by HPSEC-SID-ICP-MS as the summation of the amount of gadolinium in each of the three species of interest. The comparison indicated that, at a 95 % confidence level, the results for total gadolinium in all five nanoemulsions were statistically the same for both the ID-ICP-MS analysis and HPSEC-SID-ICP-MS analysis

methods. The amount of inter-species transformations, including both decomposition and formation, during the species-specific isotope dilution analysis were calculated by the Microsoft spreadsheet, and ranged from 0 – 18 %, with an average of 4 %. These results indicated that because isotopic ratios were fixed prior to the chromatographic run, by addition of readily lab-prepared enriched spike species, the SID quantification approach was able to overcome poor column recoveries and inter-species conversions, and to provide reliable measurements of total and speciated gadolinium without the need for validation of the recovery of individual species.

Conclusions

Although it has been shown that novel liposome-based nanoemulsion MRI contrast agents have improved stability in-vivo compared to existing contrast agents,[22,23] concerns remain about the possibility of the release of gadolinium into the body by transmetallation or instability. In the present work, two isocratic liquid chromatography methods were developed for gadolinium speciation measurements in nanoemulsion-based MRI contrast agent formulations. The RP-HPLC method was readily coupled to either fluorescence detection or online ID-ICP-MS detection for analysis of the free gadolinium and Gd-DTPA species, but the Gd-DTPA-PE species was irreversibly bound to the column, so mass balance was not achieved. The alternate HPSEC method was used to simultaneously separate and quantify free gadolinium, Gd-DTPA, and Gd-DTPA-PE. The use of triple-spike species-specific isotope dilution analysis in combination with the HPSEC separation method indicated that for the NCL formulations, the majority of the gadolinium was chelated as Gd-DTPA-PE, but the samples did contain relatively small amounts of free gadolinium and Gd-DTPA. Also, the SID approach was able to track inter-species conversions and compensate for significant on-column losses. Therefore, multiple-

spike SID-ICP-MS should be applied when reliable measurements of total and speciated gadolinium are required. However, the RP-HPLC-FL, RP-HPLC-ID-ICP-MS, and HPSEC-ID-ICP-MS techniques may prove useful to the clinical chemistry community as rapid, qualitative methods for the screening of samples for the presence of free gadolinium.

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Tables

Table 1. Composition of nanoemulsion samples NCL 55-4, NCL 56-3, NCL 57-2, NCL 58-2, and NCL 59-2.

Component	Function	NCL 55-4	NCL 56-3	NCL 57-2	NCL 58-2	NCL 59-2
egg phosphatidylcholine (Lipoid E-80)	primary emulsifier	120 mg	120 mg	120 mg	120 mg	120 mg
stearylamine	co-surfactant	40 mg	40 mg	40 mg	40 mg	40 mg
Gd-DTPA-PE	contrast agent	---	100 mg	100 mg	100 mg	100 mg
paclitaxel	biomarker	---	---	2.13 mg	---	2.13 mg
ceramide-C6	biomarker	---	---	---	10 mg	10 mg
flaxseed oil	oil phase	1 mL	1 mL	1 mL	1 mL	1 mL
purified water	aqueous phase	4 mL	4 mL	4 mL	4 mL	4 mL

Table 2. Hyphenated methods for the separation of free and bound gadolinium species.

Method designation	Analytical column	Mobile phase	Flow rate (mL min^{-1})	Detection/quantification method	Species that can be quantified
RP-HPLC-FL	Synergi Max-RP C12	50 mM Tris + 2 mM EDTA, pH 7.4	1.0	fluorescence (280 nm excitation, 316 nm emission)	free, Gd-DTPA
RP-HPLC-ID-ICP-MS	Synergi Max-RP C12	50 mM Tris + 2 mM EDTA, pH 7.4	1.0	online ID-ICP-MS	free, Gd-DTPA
HPSEC-ID-ICP-MS	BioSep SEC-S 2000	17 mM SDS + 2 mM EDTA, pH 7	0.7	online ID-ICP-MS	free, Gd-DTPA, Gd-DTPA-PE, total by summation of three individual species
HPSEC-SID-ICP-MS	BioSep SEC-S 20000	17 mM SDS + 2 mM EDTA, pH 7	0.7	SID-ICP-MS	free, Gd-DTPA, Gd-DTPA-PE, total by summation of three individual species
ID-ICP-MS	none	none	none	dilution of unseparated sample in acid, followed by ID-ICP-MS	total Gd

Table 3. Comparison of the total amounts of gadolinium in NCL nanoemulsions, determined by ID-ICP-MS on three occasions.

Nanoemulsion label	*September 2007* Measured concentration of total Gd ± expanded uncertainty ($\mu g\ g^{-1}$)	*January 2009* Measured concentration of total Gd ± expanded uncertainty ($\mu g\ g^{-1}$)	*July 2009* Measured concentration of total Gd ± expanded uncertainty ($\mu g\ g^{-1}$)
NCL 55-4	0.034 ± 0.010	0.020 ± 0.043	0.021 ± 0.018
NCL 56-3	538 ± 11	585 ± 13	592 ± 12
NCL 57-2	474 ± 10	546 ± 12	532 ± 11
NCL 58-2	404 ± 8	436 ± 10	444 ± 9
NCL 59-2	439 ± 9	494 ± 11	498 ± 10

Table 4. Summary of components of uncertainty for total and speciated gadolinium in NCL nanoemulsions.

Source	Basis	Type	Degrees of Freedom
Sample measurement repeatability	Standard uncertainty of sample measurements based on replicate measurements	A	2
Calibration of isotopic spike	Standard uncertainty of the spike calibration measurements using four independently prepared calibration mixes	A	2
Spike calibrant	Standard uncertainty of the concentration of the primary calibrant, SRM 3118a from SRM Certificate of Analysis (0.15 % relative)	B	infinite
Correction for ICP-MS instrument mass discrimination	Standard uncertainty of instrument mass discrimination correction and temporal drift correction. Estimated at 0.1 % relative	B	infinite
Weighing uncertainty	Standard uncertainty of calibration, drift (temporal and electrostatic) and relative impact on weighing measurements. Estimated at 0.01 % relative	B	infinite

Table 5. Comparison of the amounts of gadolinium quantified per species by RP-HPLC-FL and RP-HPLC-ID-ICP-MS.

	Detection/quantification method:	*RP-HPLC-FL*	*RP-HPLC-ID-ICP-MS*
Nanoemulsion label	**Species**	**Measured concentration of Gd ± expanded uncertainty ($\mu g\ g^{-1}$)**	**Measured concentration of Gd ± expanded uncertainty ($\mu g\ g^{-1}$)**
NCL 55-4	Gd-DTPA-PE	irreversibly bound to column	irreversibly bound to column
	Gd-DTPA	2.24 ± 0.88	1.95 ± 0.32
	Gd-EDTA	1.22 ± 0.48	1.22 ± 0.19
	total Gd (summation of 3 species)	mass balance not achieved	mass balance not achieved
NCL 56-3	Gd-DTPA-PE	irreversibly bound to column	irreversibly bound to column
	Gd-DTPA	58.6 ± 22. 9	16.6 ± 2.7
	Gd-EDTA	121 ± 47	34.4 ± 5.6
	total Gd (summation of 3 species)	mass balance not achieved	mass balance not achieved
NCL 57-2	Gd-DTPA-PE	irreversibly bound to column	irreversibly bound to column
	Gd-DTPA	43.3 ± 16.9	15.4 ± 2.5
	Gd-EDTA	13.9 ± 5.4	5.50 ± 0.89
	total Gd (summation of 3 species)	mass balance not achieved	mass balance not achieved
NCL 58-2	Gd-DTPA-PE	irreversibly bound to column	irreversibly bound to column
	Gd-DTPA	44.1 ± 17.2	16.5 ± 2.7
	Gd-EDTA	21.7 ± 8.5	3.37 ± 0.55
	total Gd (summation of 3 species)	mass balance not achieved	mass balance not achieved
NCL 59-2	Gd-DTPA-PE	irreversibly bound to column	irreversibly bound to column
	Gd-DTPA	65.6 ± 25.7	14.3 ± 2.3
	Gd-EDTA	11.8 ± 4.6	5.15 ± 0.83
	total Gd (summation of 3 species)	mass balance not achieved	mass balance not achieved

Table 6. Determination of the amounts of gadolinium per species and total gadolinium by HPSEC-ID-ICP-MS and HPSEC-SID-ICP-MS.

	Detection/quantification method:	*HPSEC-ID-ICP-MS*	*HPSEC-SID-ICP-MS*
Nanoemulsion label	**Species**	**Measured concentration of Gd ± expanded uncertainty ($\mu g\ g^{-1}$)**	**Measured concentration of Gd ± expanded uncertainty ($\mu g\ g^{-1}$)**
NCL 55-4	Gd-DTPA-PE	0.68 ± 0.02	0.03 ± 0.01
	Gd-DTPA	0.092 ± 0.001	0.05 ± 0.01
	Gd-EDTA	0.26 ± 0.01	0.24 ± 0.04
	total Gd (summation of 3 species)	1.03 ± 0.02	0.32 ± 0.20
NCL 56-3	Gd-DTPA-PE	86.6 ± 2.1	513 ± 34
	Gd-DTPA	13.7 ± 0.3	45.4 ± 3.0
	Gd-EDTA	47.0 ± 1.1	110 ± 7
	total Gd (summation of 3 species)	147 ± 3	669 ± 44
NCL 57-2	Gd-DTPA-PE	92.5 ± 2.2	507 ± 33
	Gd-DTPA	11.4 ± 0.3	30.3 ± 2.0
	Gd-EDTA	20.7 ± 0.5	23.4 ± 1.5
	total Gd (summation of 3 species)	125 ± 3	561 ± 37
NCL 58-2	Gd-DTPA-PE	67.1 ± 1.6	437 ± 29
	Gd-DTPA	10.9 ± 0.3	30.6 ± 2.0
	Gd-EDTA	14.6 ± 0.4	18.4 ± 1.2
	total Gd (summation of 3 species)	92.6 ± 2.2	486 ± 32
NCL 59-2	Gd-DTPA-PE	88.4 ± 2.1	491 ± 32
	Gd-DTPA	11.7 ± 1.6	30.1 ± 2.0
	Gd-EDTA	20.1 ± 0.5	11.2 ± 0.7
	total Gd (summation of 3 species)	120 ± 3	533 ± 35

Figure Captions

Figure 1: Experimental arrangements for (A) speciation and quantification of gadolinium by fluorescence detection immediately followed by online ID-ICP-MS, and (B) speciation and quantification of gadolinium by triple-spike SID-ICP-MS. PEEK tubing was used for all connections. The fluorescence detector had an excitation wavelength of 280 nm and an emission wavelength of 316 nm. A stainless steel tee was used to mix the column eluent with a stream of an enriched ^{157}Gd isotopic spike for online isotope dilution analysis. As shown in Panel B for SID analysis, the column eluent was introduced directly into the ICP-MS for detection, since the enriched gadolinium isotopes had been added directly into the sample.

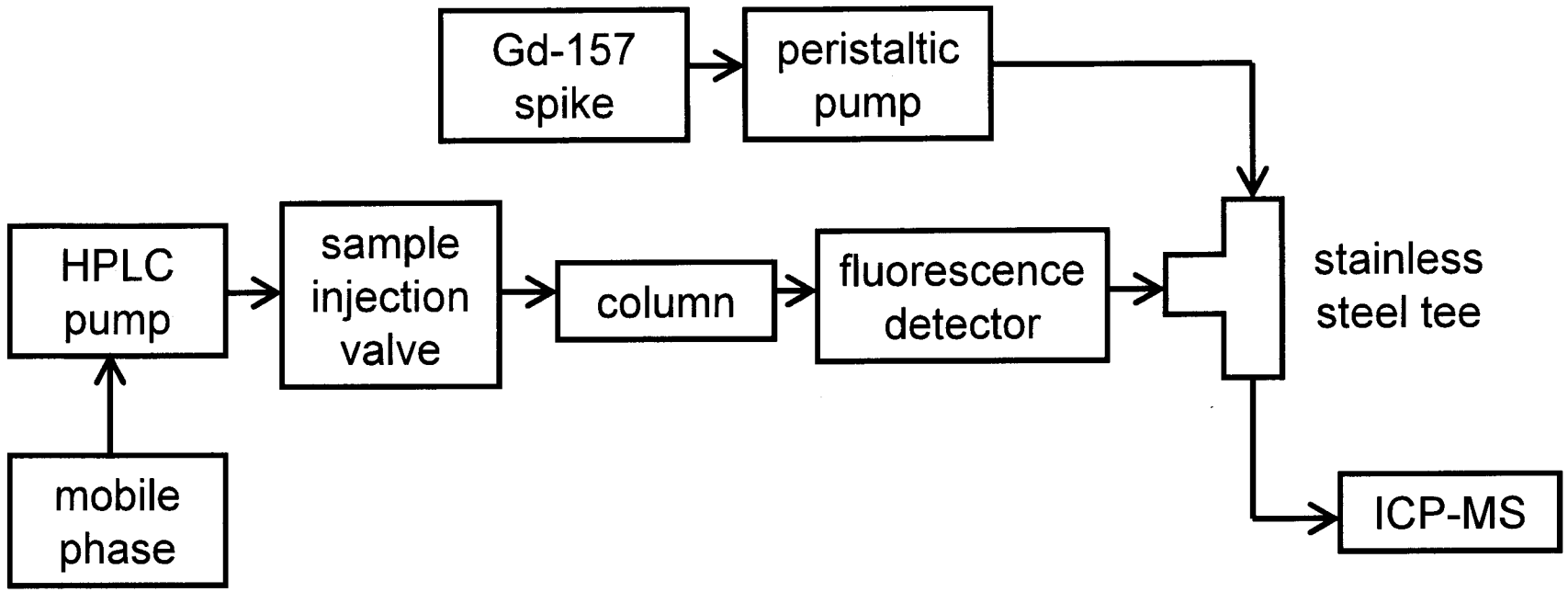
Figure 2: Overlaid fluorescence spectra following reversed-phase separations for (A) standard solutions and (B) the NCL nanoemulsion samples. Free gadolinium eluted as the Gd-EDTA species at approximately 3 min. The Gd-DTPA species eluted at about 5 min, followed by the emulsifier, Lipoid E-80, at around 6 min. The Gd-DTPA-PE species was irreversibly retained on the column, probably due to the hydrophobicity of the PE moiety. The spectra have been offset for clarity.

Figure 3: Typical ICP-MS chromatogram obtained during online isotope dilution analysis following reversed-phase separation of nanoemulsion NCL 56-3. Changes in the isotopic ratios measured by the ICP-MS as the gadolinium species eluted were used to quantify the amount of gadolinium per species. See text for details.

Figure 4: Examples of overlaid HPSEC-ICP-MS chromatograms for natural standard gadolinium species compared to nanoemulsion NCL 59-2. The ICP-MS was set to monitor mass 158 for gadolinium. These results indicated that NCL 59-2 contained the Gd-DTPA-PE species, as well as some Gd-DTPA and free gadolinium.

Figure 1

(A)


Gd-157 spike
peristaltic pump
HPLC pump
sample injection valve
column
fluorescence detector
stainless steel tee
mobile phase
ICP-MS

(B)

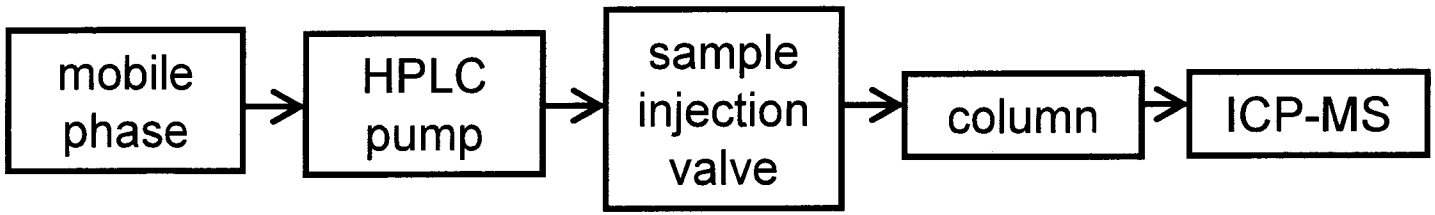

mobile phase
HPLC pump
sample injection valve
column
ICP-MS

Figure 2

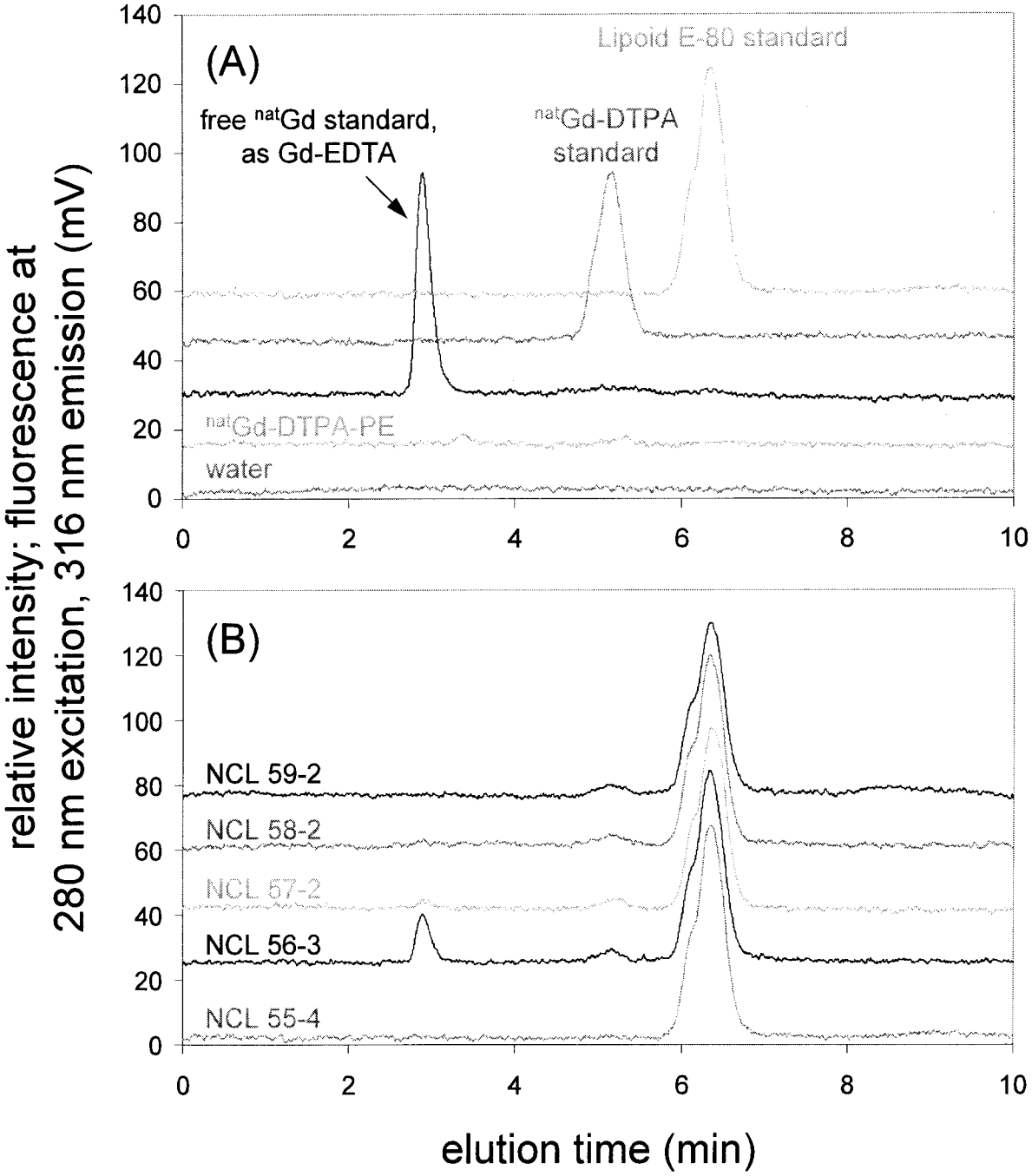

(A)
Lipoid E-80 standard
free natGd standard, as Gd-EDTA
natGd-DTPA standard
natGd-DTPA-PE
water
(B)
NCL 59-2
NCL 58-2
NCL 57-2
NCL 56-3
NCL 55-4
relative intensity; fluorescence at 280 nm excitation, 316 nm emission (mV)
elution time (min)
0
20
40
60
80
100
120
140
0
2
4
6
8
10

Figure 3

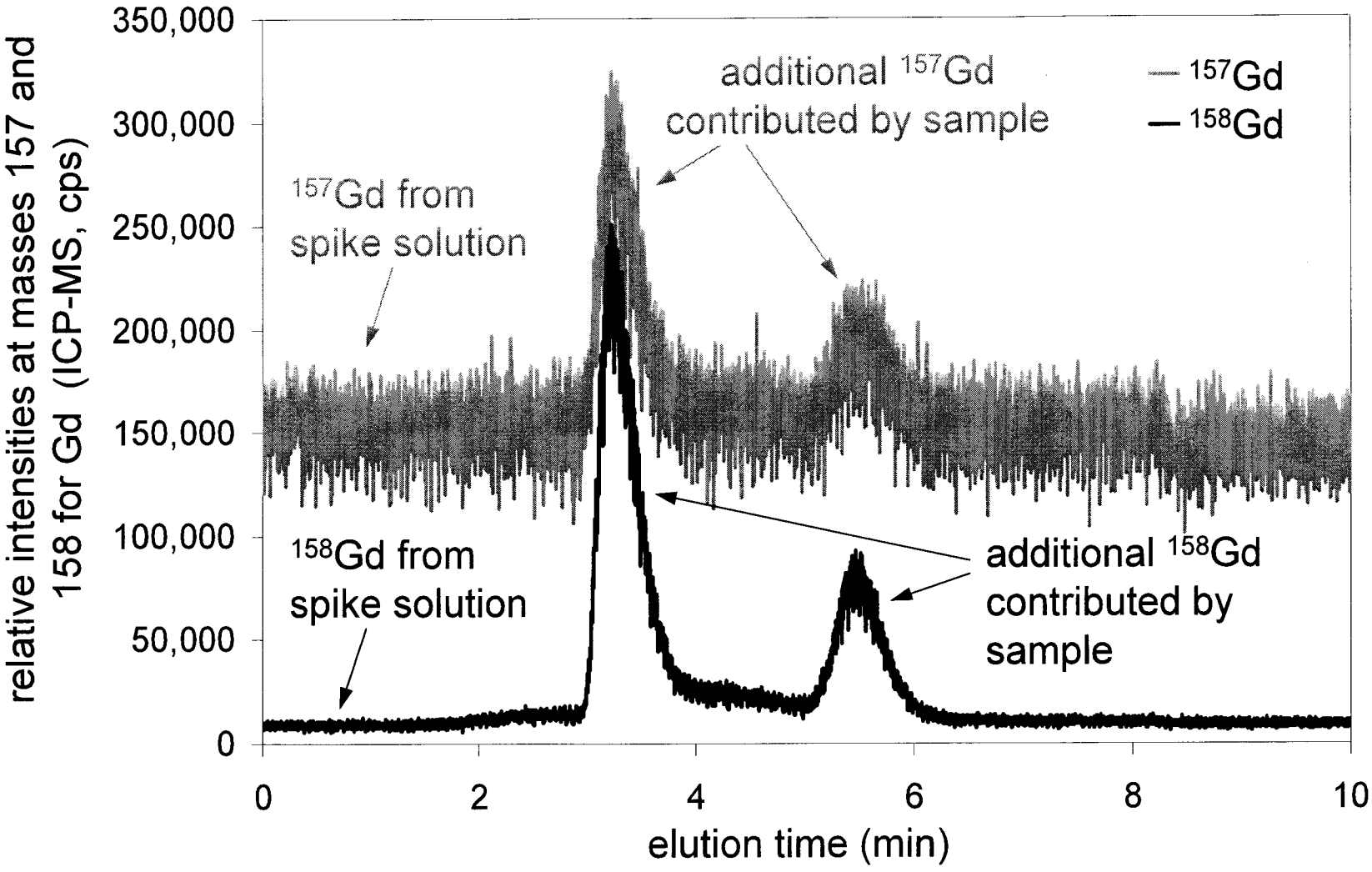
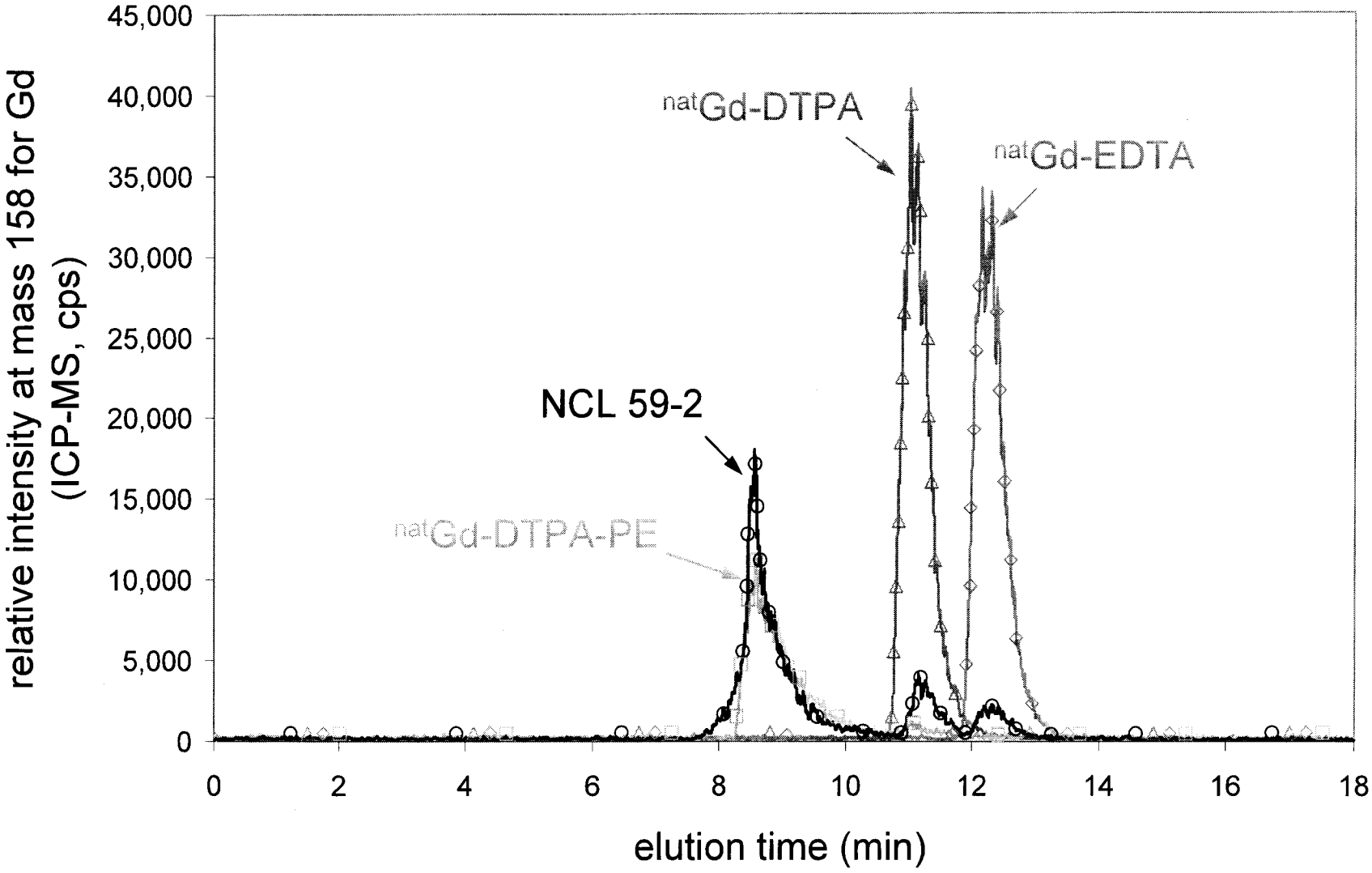

relative intensities at masses 157 and 158 for Gd (ICP-MS, cps)
350,000
300,000
250,000
200,000
150,000
100,000
50,000
0
0
2
4
6
8
10
elution time (min)
additional 157Gd contributed by sample
157Gd from spike solution
158Gd from spike solution
additional 158Gd contributed by sample
157Gd
158Gd

Figure 4


relative intensity at mass 158 for Gd (ICP-MS, cps)
45,000
40,000
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
0
2
4
6
8
10
12
14
16
18
elution time (min)
natGd-DTPA
natGd-EDTA
NCL 59-2
natGd-DTPA-PE