NIST - NCL Joint Assay Protocol, PCC-14

Quantification of Free and Chelated Gadolinium Species in Nanoemulsion-Based Magnetic Resonance Imaging Contrast Agent Formulations using Hyphenated Chromatography Methods

National Institute of Standards and Technology
Chemical Science and Technology Laboratory
Gaithersburg, MD 20899

Nanotechnology Characterization Laboratory
Frederick National Laboratory for Cancer Research
Leidos Biomedical Research, Inc.
Frederick, MD 21702
(301) 846-6939
ncl@mail.nih.gov
http://www.ncl.cancer.gov

This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

Method written by:

Danielle Cleveland
National Institute of Standards and Technology

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1. Introduction

Gadolinium-based magnetic resonance imaging (MRI) contrast agent (CA) formulations have been routinely applied in the clinical setting to improve differentiation among neighboring tissues of interest. 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. Nanoemulsion-based CAs, which use liposomal ligands to chelate the gadolinium, have shown promise as highly potent contrast agents, due to their accommodation of a significantly higher gadolinium payload, improved relaxivities, and reduced incidents of release of gadolinium ions compared to traditional CAs. With the advent of these new types of gadolinium-ligand complexes, it remains crucial to measure the amounts of total, bound, and uncomplexed gadolinium in CA formulations. This technical procedure defines the analytical protocols for gadolinium speciation measurements in liposomal MRI CA formulations that contain emulsifiers, surfactants, and therapeutic agents.

2. Reagents and Equipment [a]

CAUTION: PERSONAL PROTECTION EQUIPMENT SUCH AS SAFETY GOGGLES, LAB COAT, AND RUBBER GLOVES (LATEX OR NITRILE) MUST BE USED WHEN OPERATING UNDER THIS PROTOCOL.

2.1 Reagents

- 2.1.1 Deionized water, specific resistivity 18 MΩ·cm
- 2.1.2 Concentrated high purity hydrochloric acid (e.g. TraceMetal grade, Fisher Scientific, Pittsburgh, PA)
- 2.1.2 Concentrated high purity nitric acid (e.g. TraceMetal grade, Fisher Scientific, Pittsburgh, PA)
- 2.1.4 Ethylenediaminetetraacetic acid (EDTA) (e.g. O2793-500, Fisher Scientific, Pittsburgh, PA)

[a] Certain commercial equipment, instruments, or materials are identified in this procedure to specify adequately the experimental procedure. 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.

2.1.5 Diethylene triamine pentaacetic acid (DTPA) (e.g. D6518, Sigma-Aldrich, St. Louis, MO)

2.1.6 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid (DTPA-PE) (e.g. 790535P, Avanti Polar Lipids, Alabaster, AL)

2.1.7 Lipoid E80 (Lipoid GMBH, Ludwigshafen, Germany)

2.1.8 Sodium dodecyl sulfate (SDS) (e.g. 03945-1, Polysciences, Inc., Warrington, PA)

2.1.9 HPLC-grade methanol (e.g. 9093-03, Mallinckrodt Baker, Inc., Phillipsburg, NJ)

2.1.10 Tris (e.g. tris(hydroxymethyl)aminomethane, 75831, GFS Chemicals, Inc. Powell, OH)

2.1.11 Gadolinium standard solution (NIST SRM 3118a) [natural ratios, no enrichment, abbreviated as ^{nat}Gd]

2.1.12 Enriched gadolinium-155 (e.g. 99.82 %, Oak Ridge National Laboratory, Oak Ridge, TN)

2.1.13 Enriched gadolinium-156 (e.g. 95.50 %, Trace Sciences International, Richmond Hill, ON)

2.1.14 Enriched gadolinium-157 (e.g. 88.63 %, Oak Ridge National Laboratory, Oak Ridge, TN)

2.1.15 Enriched gadolinium-160 (e.g. 98.40 %, Trace Sciences International, Richmond Hill, ON)

2.1.16 Europium standard solution (NIST SRM 3117a), or other element, for use as internal standard, if desired

2.1.17 Ammonium hydroxide (e.g. 320145, Sigma-Aldrich, St. Louis, MO)

2.2 Equipment

2.2.1 A quadrupole-based inductively coupled plasma mass spectrometer (ICP-MS) (e.g. Plasmaquad 3, VG Elemental, Winsford, Cheshire, UK) equipped with a platinum-tipped sampler cone and a nickel skimmer cone, a standard flow glass concentric nebulizer, and a water-cooled (4ºC) spray

chamber, operated at 1350 W with standard gas flows, to serve as a gadolinium-specific detector.

2.2.2 A high pressure liquid chromatography (HPLC) pump (e.g. Varian 9012, Varian, Walnut Creek, CA), equipped with polyetheretherketone (PEEK) connections, for delivery of isocratic and gradient mobile phases, with at least 2 pump heads, capable of flow rates of 0.7 and 1.0 mL/min to deliver the desired mobile phase to the column, and to sweep the sample from the sample loop into the column.

2.2.3 A C12 reversed-phase HPLC column (250 mm x 4.6 mm id, 4 μm particle size, trimethylsilane (TMS) end-capped; e.g. Synergi Max-RP, Phenomenex, Inc., Torrance, CA), used at ambient temperature, for analytical separations.

2.2.4 A high pressure size exclusion chromatography (HPSEC) column with an exclusion range of 200 Da to 75,000 Da (300 mm x 7.80 mm, 5 μm particle size; e.g. BioSep-SEC-S2000, Phenomenex, Torrance, CA), used at ambient temperature, for analytical separations.

2.2.5 A manual injection port (e.g. Rheodyne, model 7125, Cotati, CA) equipped with a 10 μL sample loop, and LC syringe to introduce the sample to the column(s).

2.2.6 A fluorescence detector (e.g. Jasco FP-1520 Intelligent Fluorescence Detector, Jasco, Inc., Easton, MD) with an adjustable gain setting, an excitation wavelength of 280 nm and an emission wavelength of 316 nm for collection of fluorescence signals.

2.2.7 A high-purity water generation system for generating house 18 MΩ·cm deionized water.

2.2.8 A five-place analytical balance for weighing during the preparation of samples and mobile phases. The balance should be used in accordance with the procedures described in Appendix B.

2.2.9 A pH meter, three-point calibrated using standard solutions with pH of 4, 7, and 10, to measure and adjust the pH of samples and mobile phases as required.

2.2.10 A mini-vortexer for mixing of samples during sample preparation.

2.2.11 A refrigerator (4°C) for sample storage before, during, and after analyses.

2.2.12 A peristaltic pump and a tee connector (e.g. stainless steel, Teflon, other suitable material) for delivery of enriched isotopic spike during online isotope dilution-inductively couple plasma-mass spectrometry (ID-ICP-MS).

2.2.13 A PEEK connection line between the column output and the ICP-MS nebulizer.

2.2.14 An analog-to-digital converter and software (e.g. Advanced Computer Interface and AI-450 software, Dionex Corporation, Sunnyvale, CA) for fluorescence data collection.

3. Reagent Preparation

3.1 <u>50 mM Tris (pH 7.4), matrix for spike preparation</u>

Prepare in 18 MΩ·cm deionized water; shake well to dissolve Tris; adjust pH to 7.4 using hydrochloric acid and/or ammonium hydroxide as required.

3.2 <u>50 mM Tris + 2 mM EDTA (pH 7.4), RP-HPLC mobile phase</u>

Prepare in 18 MΩ·cm deionized water; shake well to dissolve Tris and EDTA; adjust pH to 7.4 using hydrochloric acid and/or ammonium hydroxide as required.

3.3 <u>17 mM SDS + 2 mM EDTA (pH 7.0), HPSEC mobile phase</u>

Prepare in 18 MΩ·cm deionized water; adjust pH to 7.4 using hydrochloric acid and/or ammonium hydroxide, as required. Avoid foaming of SDS by slow addition of water down side of vessel; minimal agitation only; some heating may be required to fully dissolve SDS.

3.4 <u>Stock, acidified ^{155}Gd solution, 1000 µg/g, gadolinium source for SID spike</u>

Prepare in 2 % volume fraction nitric acid.

3.5 <u>Stock, acidified ^{156}Gd solution, 1000 µg/g, gadolinium source for SID spike</u>

Prepare in 2 % volume fraction nitric acid.

3.6 <u>Stock, acidified ^{157}Gd solution, 1000 µg/g, gadolinium source for SID spike</u>

Prepare in 2 % volume fraction nitric acid.

3.7 <u>Stock, acidified ^{160}Gd solution, 1000 µg/g, gadolinium source for SID spike</u>

Prepare in 2 % volume fraction nitric acid.

3.8 EDTA in 50 mM Tris solution, chelating solution for SID spike

Add solid to previously-prepared Tris solution. Agitate and/or refrigerate overnight to aid dissolution. May require pH adjustment for full dissolution.

3.9 DTPA in 50 mM Tris solution, chelating solution for SID spike

Add solid to previously-prepared Tris solution. Agitate and/or refrigerate overnight to aid dissolution. May require pH adjustment for full dissolution.

3.10 DTPA-PE in 50 mM Tris solution, chelating solution for SID spike

Add solid to previously-prepared Tris solution. Agitate and/or refrigerate overnight to aid dissolution. May require pH adjustment for full dissolution.

3.11 ^{155}Gd-DTPA-PE spike solution, SID spike

Add equimolar amount of ^{155}Gd stock solution to DTPA-PE chelating solution. Adjust to pH 7.4. Suitable final concentration of gadolinium is approximately 150 μg/g.

3.12 ^{156}Gd-DTPA spike solution, SID spike

Add equimolar amount of ^{156}Gd stock solution to DTPA chelating solution. Adjust to pH 7.4. Suitable final concentration of gadolinium is approximately 150 μg/g.

3.13 ^{160}Gd-EDTA spike solution, SID spike

Add equimolar amount of ^{160}Gd stock solution to EDTA chelating solution. Adjust to pH 7.4. Suitable final concentration of gadolinium is approximately 150 μg/g.

3.14 ^{nat}Gd-DTPA-PE solution, natural sample for mass discrimination corrections

Add equimolar amount of ^{nat}Gd stock solution to DTPA-PE chelating solution. Adjust to pH 7.4. Suitable final concentration of gadolinium is approximately 150 μg/g.

3.15 ^{nat}Gd-DTPA solution, natural sample for mass discrimination corrections

Add equimolar amount of ^{nat}Gd stock solution to DTPA chelating solution. Adjust to pH 7.4. Suitable final concentration of gadolinium is approximately 150 μg/g.

3.16 ^{nat}Gd-EDTA solution, natural sample for mass discrimination corrections

Add equimolar amount of ^{nat}Gd stock solution to EDTA chelating solution. Adjust to pH 7.4. Suitable final concentration of gadolinium is approximately 150 µg/g.

4. Measurement of Total Gadolinium by ID-ICP-MS

4.1 Instrumentation

4.1.1 Prepare the ICP-MS by cleaning existing or installing new sampler and skimmer cones, plasma torch, spray chamber, nebulizer, and pump tubing.

4.1.2 Turn the instrument on, and allow 15-30 minutes for warm-up. See manufacturer's recommendation for start-up procedures.

4.1.3 Verify that the instrument is optimized and working satisfactorily by assessment of performance parameters (e.g. sensitivity, background, oxide ratios, etc.) and by alteration of parameters such as ion lens voltages, plasma gas flows, and torch position.

4.1.4 Check for the presence of spectral interferences (e.g. oxides, doubly charged, etc.) for the elements and isotopes of interest in the matrix of interest (e.g. acidified, nitric acid) by comparison of the measured isotopic ratios in the natural sample to the ratios for a known isotopic standard. A list of potential interfering species for gadolinium is given below, in Table 1, by gadolinium isotopic mass.

4.1.5 Reduce or eliminate interferences, as required, using appropriate method(s) (e.g. collision gas, separations, correction algorithm, etc.).

4.1.6 Samples may be introduced into the ICP-MS by use of auto-sampler, or manually. Care should be taken to avoid contamination of the samples.

Table 1. List of possible isobaric interferences for gadolinium

Mass 152	Mass 154	Mass 155	Mass 156	Mass 157	Mass 158	Mass 160
Sm	Sm	BaOH	Dy	PrO	Dy	Dy
BaOH	BaOH	LaO	CeO	CeOH	CeO	NdO
BaO	BaO	CeOH			NdO	SmO
CeO	CeO					NdOH
	LaO					

4.2 Sample Preparation and Data Analysis

Measurements of total gadolinium may be made by isotope dilution analysis (IDA) or by external calibration using ICP-MS, based on the analyst's preference. In order to perform accurate isotope dilution analysis, the analyst needs to have a general idea of the amount of total gadolinium in the sample. If this information has not been provided, total gadolinium in the sample may be measured by ICP-MS with external calibration, or another method of choice. If the amount of gadolinium is known, or has been estimated, the analyst may proceed to Step 4.2.6. Steps 4.2.1 – 4.2.5 describe the use of external calibration. An internal standard should be used with external calibration if isotope dilution analysis will not be performed.

4.2.1 Prepare a suite of gadolinium calibration standards by serial dilution of a primary standard (e.g. NIST SRM 3118a, Gadolinium Standard Solution). If IDA will not be used, an internal standard, such as europium, should be added to each calibration standard. A stock standard of the internal standard should be prepared by serial dilution of a primary standard (e.g. NIST SRM 3117a, Europium Standard Solution). The final concentration of europium in each standard should be such that the ICP-MS detector response ranges from 400,000 to 800,000 counts per second (e.g. 5 ng/g). Include a blank solution that contains only the internal standard for external calibration.

4.2.2 Mix the nanoemulsion samples well prior to use, with a mini-vortexer, in order to create a homogeneous mixture. It should be noted that the nanoemulsions may begin to separate over time.

4.2.3 Aliquots of the nanoemulsion samples can be diluted as required in 2 % volume fraction nitric acid, or other dilute acid mixtures (e.g. 3 % volume fraction HCl + 1 % volume fraction HNO_3) as desired. Shake well. If IDA will not be used, add the internal standard to each sample, such that the final concentration of europium creates a detector response between 400,000 and 800,000 counts per second (e.g. 5 ng/g).

4.2.4 Measure the total amount of gadolinium in each unknown sample by ICP-MS. Use the ICP-MS to make 5 replicate measurements of 30 seconds each, per sample, with dwell times of 10 ms per isotope at two major gadolinium isotopes (e.g. 157 and 158).

4.2.5 Export the ICP-MS data for manipulation and creation of a calibration curve. Correct the raw data as required for relative abundances and the response of the blank sample. If IDA is not being used, also normalize the data for the concentration response of the internal standard. Plot the corrected, average counts of gadolinium as a function of concentration for each calibration standard, and perform a linear regression to obtain the equation of the line. The equation can then be used to calculate the final concentration of gadolinium that was in each *diluted* sample. Back calculate using the dilution equation ($C_1V_1 = C_2V_2$, where C denote gadolinium concentration, and V denotes volume, and 1 and 2 denote native sample and diluted ICP-MS sample solution, respectively) to determine the amount of total gadolinium in the original sample. For more information, see reference **(1)**. The results can now be used for sample preparation for IDA, if desired.

Isotope dilution analysis is performed by addition of a known amount of an isotopic spike solution with a known, isotopic enrichment, to a known amount of a sample that contains the natural isotope ratios. The following procedure, Steps 4.2 6 – 4.2.11, for total gadolinium is optional.

4.2.6 Prepare stock and working standards of natural gadolinium and ^{157}Gd enriched isotopic spike in 2 % volume fraction nitric acid. In particular, the natural gadolinium solution should be carefully prepared from a primary standard (e.g. SRM 3118a). Enriched isotopes can be obtained from commercial vendors (e.g. Oak Ridge National Laboratory, Oak Ridge, TN).

4.2.7 In order to reduce error magnification effects, an isotopic ratio of the spike/reference isotopes between 3 and 8 (for gadolinium only, $^{157}Gd/^{158}Gd$) will be used. Other metals or isotope pairings may require other ratios. Prepare calibration mixtures of ^{nat}Gd and ^{157}Gd spike with a ratio between 3 and 8, by mixing appropriate volumes of the ^{nat}Gd and ^{157}Gd stock standards.

4.2.8 Dilute to volumes and concentrations appropriate for ICP-MS using 2 % volume fraction nitric acid. These mixtures will be used to calibrate the ^{157}Gd spike solution.

4.2.9 Prepare the nanoemulsion samples by addition of an appropriate volume of the enriched isotopic ^{157}Gd spike to appropriate volumes of samples, and dilute with 2 % volume fraction nitric acid for ICP-MS analysis.

4.2.10 Measure the $^{157}Gd/^{158}Gd$ ratio in each sample and calibration mix using the ICP-MS, operated at optimized conditions.

4.2.11 Calculate the concentration of total gadolinium in the sample using Equation 1. The measured ratios should be first corrected for mass discrimination, as required. The concentration of the enriched ^{157}Gd isotope in the spike solution is determined by reverse isotope dilution analysis *via* measurements made on the calibration mixes.

$$C_{sample} = \frac{M_{spike} \times K}{W} \times \frac{A_{spike} - B_{spike} \times R}{B_{nat} \times R - A_{nat}} \qquad \text{Eq.1}$$

where:

C_{sample} = concentration (mass fraction) of analyte in sample (µg/g)

M_{spike} = mass of isotopic spike (µg)

K = ratio of natural/spike atomic weights

W = sample weight (g)

A_{spike} = isotopic abundance of the reference isotope in the spike solution

B_{spike} = isotopic abundance of spike isotope in the spike solution

A_{nat} = natural isotopic abundance of the reference isotope

B_{nat} = natural isotopic abundance of spike isotope

R = measured isotope ratio, reference/spike

5. Reversed-Phase Liquid Chromatography with Fluorescence Detection and on-line ID-ICP-MS

5.1 Connect the C12 reversed-phase column to the HPLC pump, and attach the fluorescence detector to the column output. Increase the gain on the fluorescence detector to the highest setting (i.e. 1000 on Jasco FP-1520). For measurements by online ID-ICP-MS, a tee connector is used to mix a ^{157}Gd enriched isotope with the output of the fluorescence detector, prior to introduction in the ICP-MS. The experimental arrangement is given in Figure 1, below.

5.2 Install a mobile phase reservoir containing 100 % volume fraction methanol on one pump head, and a reservoir containing the 50 mM Tris + 2 mM EDTA buffer (pH 7.4) solution on a second pump head.

5.3 Prime the pump with both solutions in order to remove air bubbles from the lines.

5.4 Condition the column with 100 % volume fraction methanol for 5 minutes, using a flow rate of 1 mL/min.

5.5 Re-equilibrate the stationary phase by pumping the 50 mM Tris + 2 mM EDTA buffer through the column at a flow rate of 1 mL/min for 15 minutes. In this method, the chelating agent EDTA is added to the mobile phase to bind any free gadolinium ions in the samples, which results in the formation of the Gd-EDTA species. In this way, no sample preparation or pretreatment steps are required prior to sample analysis. The other species of interest, Gd-DTPA and Gd-DTPA-

PE, in the samples are stable at neutral pH and are not affected by the presence of excess EDTA.

5.6 Program a 10 minute isocratic run, with flow rate 1 mL/min, using 50 mM Tris + 2 mM EDTA as the mobile phase.

5.7 Maintain the flow rate at 1 mL/min, and load the sample into the injection port. Thoroughly wash the syringe between uses.

5.8 Simultaneously load the sample onto the column by switching the injection port, and start the LC program.

5.9 Repeat steps 5.4 and 5.5 to condition and re-equilibrate the column before running the next sample. The conditioning and re-equilibration steps can be built into the LC program, if desired.

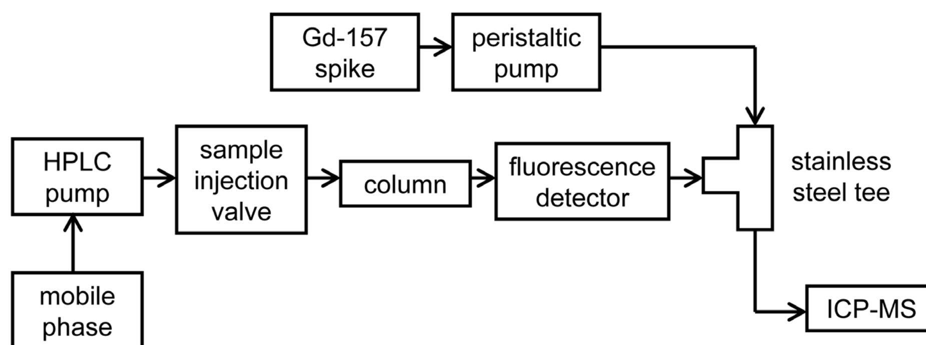

Figure 1. Experimental arrangement for reversed phase separations with fluorescence detection and online ID-ICP-MS analysis.

5.10 Collect the fluorescence signal data using an analog-to-digital converter and associated software. Start data collection at the same time as the LC method is started. Use the software to export the data (e.g. .txt, .csv files) for processing using spreadsheet software.

5.11 Prepare and analyze a suite of ^{nat}Gd-EDTA standards containing known amounts of gadolinium. The fluorescence detector will give a linear, concentration-dependent response.

5.12 Create a calibration curve to quantify the amounts of free gadolinium by plotting the area of the fluorescence signal against the concentration of gadolinium in the known ^{nat}Gd-EDTA standards.

5.13 Use the equation of the calibration line and the area of the fluorescence peak of each unknown sample to calculate the concentration of free gadolinium, as Gd-EDTA, in the samples.

5.14 Run standard solutions of ^{nat}Gd-DTPA and Lipoid E80, if desired. These species also give fluorescence signals and can be quantified by fluorescence spectroscopy, if desired. The Gd-DTPA-PE sample will be irreversibly retained on the column, and no signal will be observed. Typical retention times for each standard and examples of signals from nanoemulsion samples are given in Figure 2.

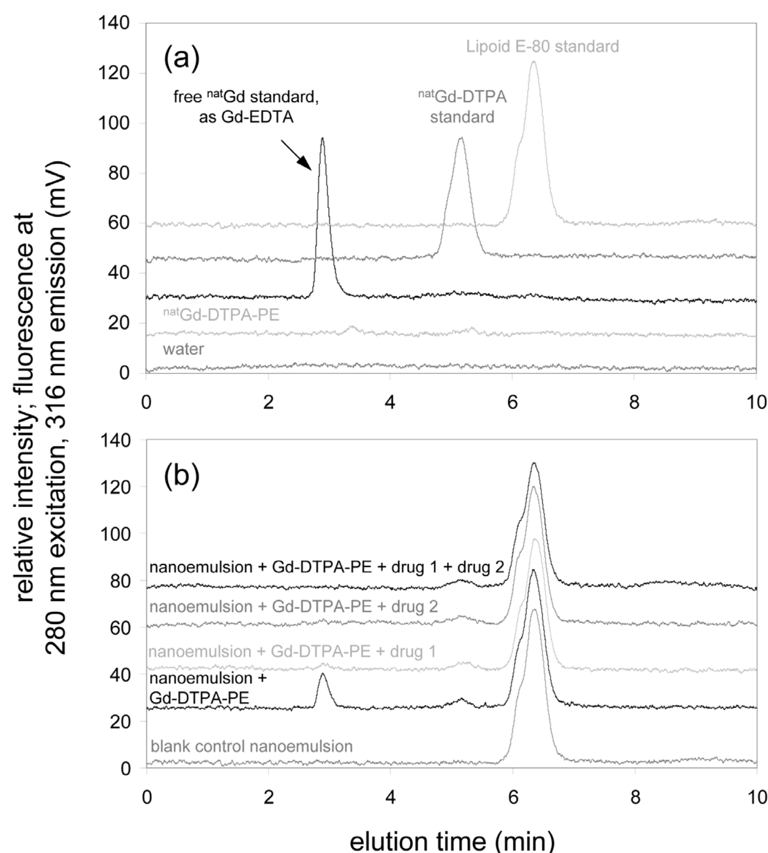

Figure 2. Examples of the fluorescence signals obtained from (A) free gadolinium, Gd-DTPA, Gd-DTPA-PE, and Lipoid E-80 standards, and (B) from real nanoemulsion samples.

5.15 To perform quantification by online ID-ICP-MS, use the peristaltic pump to deliver a solution of enriched ^{157}Gd isotopic spike, with a known concentration, to the stainless steel tee to be mixed with the column effluent. The flow rate for the spike solution should be approximately 0.5 mL/min. The ^{157}Gd solution should have a gadolinium concentration such that the $^{157}Gd/^{158}Gd$ ratio ranges from 3 to 8 (following data processing).

5.16 Set up the ICP-MS to monitor time-resolved gadolinium isotope masses 157 and 158, using a 10 ms dwell time, and a 10 minute acquisition time.

5.17 Start data acquisition on the ICP-MS at the same time the LC program is started. An example of the raw data for an unknown sample is given in Figure 3.

5.18 Export the raw data from the ICP-MS in a suitable format (i.e. .txt, .csv, etc.), and import into spreadsheet software for manipulation.

5.19 Process the data according to the procedure devised by Rottmann and Heumann for online ID-ICP-MS **(2)**.

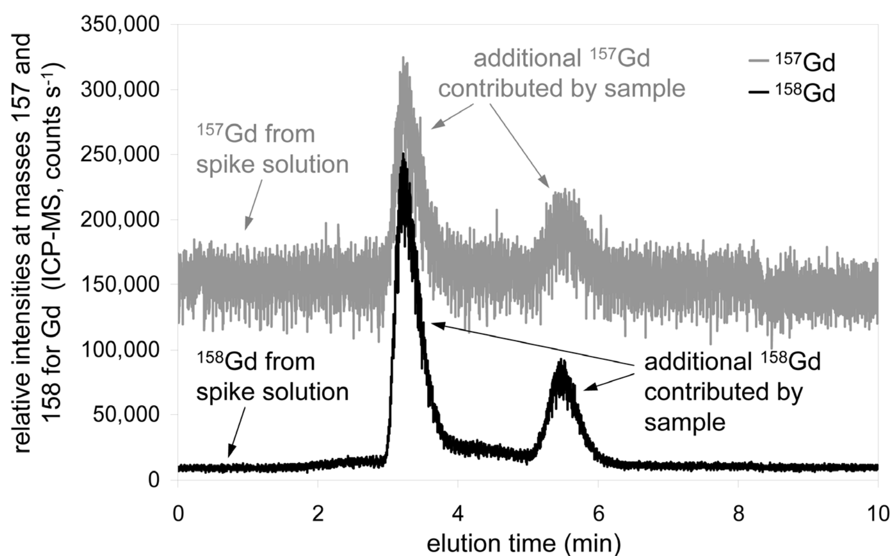

Figure 3. An example of the raw data, collected by ICP-MS, during online ID-ICP-MS analysis for a nanoemulsion + Gd-DTPA-PE sample.

6. High Pressure Size Exclusion Chromatography with Online ID-ICP-MS Detection

6.1 Connect the HPSEC column to the HPLC pump, and use a short length of tubing to connect the column output with the stainless steel tee. As before, use a peristaltic pump to mix a ^{157}Gd enriched isotope solution of appropriate concentration with the output of the HPSEC column, prior to introduction in the ICP-MS. The flow rate for the spike solution should be approximately 0.5 mL/min. The ^{157}Gd solution should have a known gadolinium concentration such that the $^{157}Gd/^{158}Gd$ ratio ranges from 3 to 8 (following data processing).

6.2 Install a mobile phase reservoir containing the 17 mM SDS + 2 mM EDTA buffer (pH 7) solution on a pump head. No conditioning or re-equilibration steps are required for the HPSEC method. As before, EDTA is added to the mobile phase to bind any free gadolinium ions in the samples directly in the sample loop, which results in the formation of the Gd-EDTA species. In this way, no sample preparation or pretreatment steps are required prior to sample analysis. The other species of interest, Gd-DTPA and Gd-DTPA-PE, in the samples are stable at neutral pH and are not affected by the presence of excess EDTA. Tailing will occur on the free gadolinium peak if EDTA is omitted.

6.3 Prime the pump with the mobile phase solution in order to remove air bubbles from the lines.

6.4 Program an 18 minute isocratic run, with flow rate 0.7 mL/min, using 17 mM Tris + 2 mM EDTA as the mobile phase.

6.5 Maintain the flow rate at 0.7 mL/min, and load the sample into the injection port. Thoroughly wash the syringe between uses.

6.6 Set up the ICP-MS to monitor time-resolved, gadolinium isotope masses 157 and 158, using a 10 ms dwell time, and an 18 minute acquisition time.

6.7 Simultaneously load the sample onto the column by switching the injection port, and start the LC program.

6.8 Start data acquisition on the ICP-MS at the same time the LC program is started.

6.9 Export the raw data from the ICP-MS in a suitable format (e.g. .txt, .csv, etc.), and import into spreadsheet software for manipulation.

6.10 An example of the resulting time-resolved chromatogram for the HPSEC column is given in Figure 4. Process the data according to the procedure devised by Rottmann and Heumann for online ID-ICP-MS **(2)**.

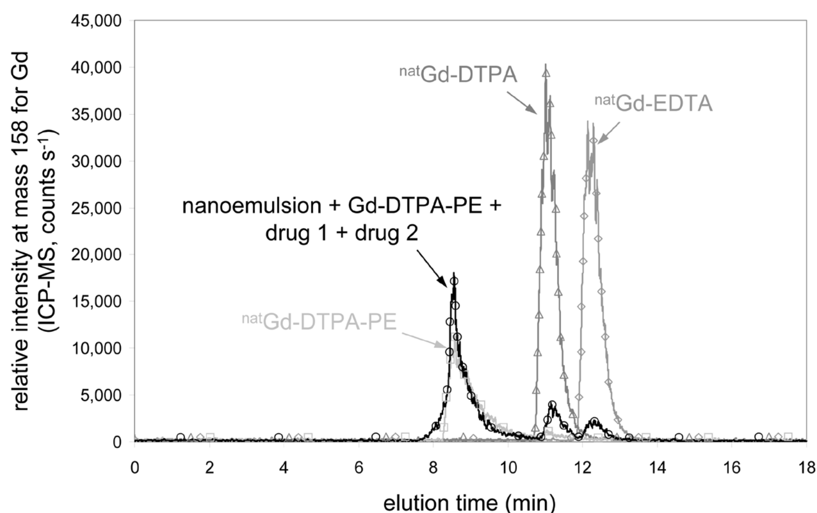

Figure 4. Example of a HPSEC separation for ^{nat}Gd-ligand standards and a nanoemulsion sample. The Gd-DTPA-PE species eluted around 9 min, while the Gd-DTPA and Gd-EDTA eluted around 11 and 12 min, respectively. The nanoemulsion sample contained all three species.

7. High-Pressure Size Exclusion Chromatography SID-ICP-MS Analysis

7.1 To prepare samples for SID analysis, add an appropriate volume of each of the three SID spike solutions (^{155}Gd-DTPA-PE, ^{156}Gd-DTPA, and ^{160}Gd-EDTA) to an aliquot of the unknown sample. The volumes of each SID spike should be such that the target ICP-MS-measured ratios range from 6 to 8 for ^{155}Gd/^{158}Gd, ^{156}Gd/^{158}Gd, and ^{160}Gd/^{158}Gd. It should be noted that this may require some trial and error, particularly for previously unknown samples. A graphical representation of this process is given in Figure 5.

7.2 Connect the HPSEC column to the HPLC pump, and couple the column effluent directly into the ICP-MS nebulizer.

7.3 Install a mobile phase reservoir containing the 17 mM SDS + 2 mM EDTA buffer (pH 7) solution on a pump head. No conditioning or re-equilibration steps are required for the HPSEC method.

7.4 Prime the pump with the mobile phase solution in order to remove air bubbles from the lines.

7.5 Program an 18 minute isocratic run, with flow rate 0.7 mL/min, using 17 mM Tris + 2 mM EDTA as the mobile phase.

7.6 Maintain the flow rate at 0.7 mL/min, and load the sample into the injection port. Thoroughly wash the syringe between uses.

7.7 Set up the ICP-MS to monitor gadolinium isotope masses 155, 156, 158, and 160 using a 10 ms dwell time, and an 18 minute acquisition time.

7.8 Simultaneously load the sample onto the column by switching the injection port, and start the LC program.

7.9 Start data acquisition on the ICP-MS at the same time the LC program is started. In addition to the unknown samples, the ^{nat}Gd-DTPA-PE, ^{nat}Gd-DTPA, and ^{nat}Gd-EDTA standards should also be analyzed, as-is and unspiked. These results will be used for mass discrimination corrections.

7.10 Export the raw data from the ICP-MS in a suitable format (e.g. .txt, .csv, etc.), and import into spreadsheet software for manipulation.

7.11 Process the time-resolved data according to the SID procedure described by Rodríguez-González, et al **(3)**. The spreadsheet should be set up to solve three unknowns in three equations.

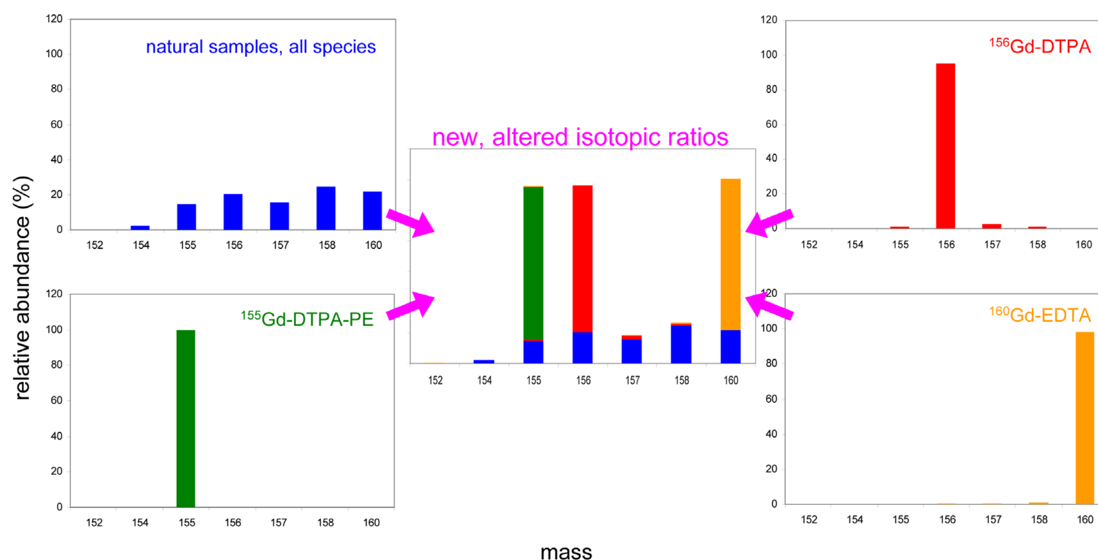

Figure 5. A graphical representation of multiple-spike species-specific isotope dilution analysis. The unknown sample contains the natural gadolinium ratio, while each of the spike species contain a different enriched gadolinium isotope. Upon mixing the sample with the spike solutions, new, altered isotopic ratios are generated. The resulting ratios are mathematically related to the masses of the samples and spikes, the abundances of the natural and altered isotope ratios, and the concentration of gadolinium in the unknown sample.

8. References

1. Daniel C. Harris, Calibration Methods, *Quantitative Chemical Analysis*, 5th ed; W. H. Freeman and Company: New York, 1999; 93-106.
2. L. Rottmann and K. G. Heumann, Development of an on-line isotope dilution technique with HPLC/ICP-MS for the accurate determination of elemental species, *Fresenius J. Anal. Chem*. **1994**, *350*, 221-227.
3. P. Rodríguez-González, J. M. Marchante-Gayón, J. I. García Alonso, and A. Sanz-Medel, Isotope dilution analysis for elemental speciation: A tutorial review, *Spectrochim. Acta, Part B*, **2005**, *60*, 151-207.

4. Guide to the Expression of Uncertainty in Measurement, 1st Edition, 1993,: corrected and reprinted edition, 1995, International Organisation for Standardisation, Geneva, Switzerland.

9. Abbreviations

CA	contrast agent
cm	centimeter
Da	Dalton
DTPA	diethylene triamine pentaacetic acid
DTPA-PE	1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid
EDTA	ethylenediaminetetraacetic acid
g	gram
Gd	gadolinium
HCl	hydrochloric acid
HNO_3	nitric acid
HPLC	high pressure liquid chromatography
HPSEC	high pressure size exclusion chromatography
ICP-MS	inductively coupled plasma
id	inner diameter
ID-ICP-MS	isotope dilution- inductively coupled plasma
IDA	isotope dilution analysis
mg	milligram
mL	milliliter
min	minute
mm	millimeter
mM	millimolar
MRI	magnetic resonance imaging
ms	millisecond
MΩ	megohm
µg	microgram
µm	micrometer
NIST	National Institute of Standards and Technology
nm	nanometer
RP-HPLC	reverse phase-high pressure liquid chromatography

SDS	sodium dodecyl sulfate
SID	speciated isotope dilution
SRM®	Standard Reference Material®
W	watt

Appendix A. Uncertainty for ICP-MS Measurements

Uncertainty: Estimate Type A and Type B components of uncertainty for the ICP-MS measurements and provide an uncertainty budget for each measurement. Examples of potential sources of uncertainty include the following: sample measurement repeatability, calibration of isotopic spike, spike calibrant, ICP-MS instrument mass discrimination, dead time, and background corrections, weighing uncertainty, and any spectral interference corrections. The uncertainty components should be combined according to ISO guidelines **(4)** to produce a combined standard uncertainty, and expressed as an expanded uncertainty at a defined level of confidence by multiplying the standard uncertainty by the coverage factor k.

Appendix B. Procedure for Day-to-Day Use of Analytical Balances

1. Inspect and adjust the level and clean the balance, if necessary. Care should be taken to turn off the power before cleaning, and to allow the balance to stabilize after restoration of power.
2. Obtain a set of NIST-traceable calibrated check masses.
3. Weigh two check masses to verify the accuracy and linearity of the balance. A static eliminator (e.g. Staticmaster Ionizer or AD-1683 Static Eliminator) may be useful.
4. For check masses greater than or equal to 50 mg, the observed differences in mass compared to the previously verified mass value should be less than 0.1 %. For masses less than 50 mg, acceptance criteria are based on the manufacturer specifications.
5. If the specifications are not met, the user should verify that the check mass has not been compromised by weighing on at least one other calibrated balance. The balance should be removed from service if no problems with the check mass are detected.

Uncertainty: The uncertainty for each mass measurement will depend on the masses determined and the mode of use (e.g. direct weighing or weighing by difference). See the manufacturer's specifications for the balance.