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Speciation Analysis of Gadolinium in the Water-Insoluble Rat Brain Fraction After Administration of Gadolinium-Based Contrast Agents

Izabela Strzeminska, PhD,*† Cécile Factor, PhD,* Philippe Robert, PhD,* Joanna Szpunar, PhD, DSc,† Claire Corot, PharmD, PhD,* and Ryszard Lobinski, PhD, DSc†‡

Purpose: To date, the analysis of gadolinium (Gd) speciation in the brain of animals administered with macrocyclic and linear Gd-based contrast agents (GBCAs) has been limited to Gd soluble in mild buffers. Under such conditions, less than 30% of the brain tissue was solubilized and the extraction recoveries of GBCAs into the aqueous phase were poor, especially in the case of the linear GBCAs. The aim of this study was to find the conditions to solubilize the brain tissue (quasi-)completely while preserving the Gd species present. The subsequent analysis using size exclusion chromatography–inductively coupled plasma–mass spectrometry (SEC-ICP-MS) was intended to shed the light on the speciation of the additionally recovered Gd.

Methods: Four groups of healthy female Sprague Dawley rats (SPF/OFA rats; Charles River, L'Arbresle, France) received randomly 5 intravenous injections (1 injection per week during 5 consecutive weeks) of either gadoterate meglumine, gadobenate dimeglumine, gadodiamide (cumulated dose of 12 mmol/kg), or no injection (control group). The animals were sacrificed 1 week (W1) after the last injection. Brain tissues were solubilized with urea solution, whereas tissues extracted with water served as controls. Total Gd concentrations were determined in the original brain tissue and its soluble and insoluble fractions by inductively coupled plasma–mass spectrometry (ICP-MS) to calculate the Gd accumulation and extraction efficiency. Size exclusion chromatography coupled to ICP-MS was used to monitor the speciation of Gd in the soluble fractions. The stability of GBCAs in the optimum conditions was monitored by spiking the brain samples from the untreated animals. The column recoveries were precisely determined in the purpose of the discrimination of weakly and strongly bound Gd complexes. The identity of the eluted species was explored by the evaluation of the molecular size and retention time matching with Gd chelates and ferritin standard. The speciation analyses were carried out for 2 different brain structures, cortex and cerebellum.

Results: The combination of water and urea extractions (sequential extraction) managed to solubilize efficiently the brain tissue (97% ± 1%) while preserving

the stability of the initially injected form of GBCA. For macrocyclic gadoterate, 97% ± 1% and 102% ± 3% of Gd initially present in the cortex and cerebellum were extracted to the soluble fraction. For gadobenate, similar amounts of Gd (49% ± 1% and 46% ± 4%) were recovered from cortex and cerebellum. For gadodiamide, 48% ± 2% of Gd was extracted from cortex and 34% ± 1% from cerebellum. These extraction efficiencies were higher than reported elsewhere. The SEC-ICP-MS and the column recovery determination proved that Gd present at low nmol/g levels in brain tissue was exclusively in the intact GBCA form in all the fractions of brain from the animals treated with gadoterate. For the linear GBCAs (gadobenate and gadodiamide), 3 Gd species of different hydrodynamic volumes were detected in the urea-soluble fraction: (1) larger than 660 kDa, (2) approximately 440 kDa, and (3) intact GBCAs. The species of 440 kDa corresponded, on the basis of the elution volume, to a Gd³⁺ complex with ferritin. Gd³⁺ was also demonstrated by SEC-ICP-MS to react with the ferritin standard in 100 mM ammonium acetate (pH 7.4). In contrast to macrocyclic gadoterate, for linear GBCAs, the column recovery was largely incomplete, suggesting the presence of free, hydrolyzed, or weakly bound Gd³⁺ with endogenous ligands.

Conclusions: The sequential extraction of rat brain tissue with water and urea solution resulted in quasi-complete solubilization of the tissue and a considerable increase in the recoveries of Gd species in comparison with previous reports. The macrocyclic gadoterate was demonstrated to remain intact in the brain 1 week after administration to rats. The linear GBCAs gadobenate and gadodiamide underwent ligand exchange reactions resulting in the presence of a series of Gd³⁺ complexes of different strength with endogenous ligands. Ferritin was identified as one of the macromolecules reacting with Gd³⁺. For the linear GBCAs, 3% of the insoluble brain tissue was found to contain more than 50% of Gd in unidentified form(s).

Key Words: gadoterate, gadobenate, gadodiamide, gadolinium speciation, water-insoluble brain fraction, gadolinium retention, SEC-ICP-MS

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Contrast enhancement in magnetic resonance imaging is usually achieved by intravenous administration of chelates of the paramagnetic gadolinium ion (Gd³⁺) with polyaminocarboxylic acids in charge of reducing its acute and chronic toxicities.¹ These chelates, referred to as Gd-based contrast agents (GBCAs), vary in terms of thermodynamic (logK_{therm} 16.6–25.6) and kinetic stabilities (T_{1/2} at pH 1 and 25°C < 5 seconds to 338 hours) and can be divided, on the basis of the molecular structure, into linear (L-GBCAs) and macrocyclic (M-GBCAs), either class of which can be further subdivided into ionic and nonionic compounds.² Under physiological conditions, the M-GBCAs are more stable than the linear type and ionic L-GBCAs more than nonionic L-GBCAs.³

Historically, the relatively hydrophilic, small-sized molecules of GBCAs were designed not to enter the cells or to penetrate the blood-brain barrier, and to be completely eliminated in the intact form.⁴ However, in the last decade, it has become well established that a trace amount of Gd does accumulate for a prolonged time in multiple organs, including the brain, after single^{5,6} and repeated GBCAs administration.^{7–10} Based on the animal studies, it can be estimated that eventually less than 0.0005% of the injected dose is found in the brain.¹¹ Although the

amount of retained Gd is at trace level, the local concentrations may be elevated, achieving as much as 64 nmol/g (10.1 ppm).¹² Albeit these observations have not been associated with any clinical effects or histological alterations, the understanding of the mechanisms of the Gd retention, including exhaustive characterization of the Gd species involved, is essential for the long-term GBCAs safety. Consequently, it has been attracting the attention of regulatory agencies such as the European Medical Agency and the Food and Drug Administration.^{13,14}

Numerous studies in patients¹⁵ and in animals¹⁶ provided evidence for the relationship between the structure of GBCAs and the amount of total Gd accumulated in tissue. Significantly higher concentrations were found in brain tissues after L-GBCAs administration in comparison with M-GBCAs. Several localization experiments demonstrated that the Gd distribution in the brain was not uniform; the elements accumulated preferentially in the dentate nucleus or globus pallidus, which are rich in endogenous metals such as iron.^{17,18} This finding suggests that the L-GBCAs may partially undergo a dechelation process through the transmetalation with endogenous metals such as iron, producing new Gd species. Transmission electron microscopy (TEM) associated with nanoSIMS (nanoscale secondary ion mass spectrometry) demonstrated the presence of Gd deposits containing phosphorus in the deep cerebellar nuclei (DCN), strongly suggesting formation of GdPO₄.¹⁹

Complementary data on the speciation of the residual Gd can be obtained by chromatography coupled with Gd-specific or GBCA-specific detection by inductively coupled plasma–mass spectrometry (ICP-MS)^{11,20} or electrospray mass spectrometry,²¹ respectively. Size exclusion chromatography (SEC), offering the separation according to the hydrodynamic volume, has been favored as the separation technique, allowing the preservation of the Gd ligand species.¹⁶ Even if low, the separation efficiency was sufficient to discriminate between the intact GBCA species and the potential complexes of Gd with other (macromolecular) ligands present in the sample.^{11,20} Indeed, high-molecular-weight (HMW) species (66 to 250 kDa, depending on the type of the column and calibration markers used) were detected but were not formally identified.^{11,20} An alternative to SEC is hydrophilic interaction liquid chromatography. It offers a better resolution for low-molecular-weight (LMW) compounds for the identification or quantification of intact GBCAs in water fraction, but it is unable to detect HMW species.

The coupled techniques have 2 significant drawbacks. First, the Gd species have to be present in solution, which raises questions about their recovery, in the original chemical form, from the tissue during sample treatment and chromatography. A typical water extraction procedure, based on the tissue homogenization with 10 mM Tris-HCl buffer (pH 7.4) and the recovery of the subsequently analyzed supernatant, shows relatively poor extraction efficiencies of Gd, which means that only a small fraction of the Gd present in the tissue is actually accounted for. Indeed, Frenzel et al¹¹ could recover approximately 17% to 37% of Gd present in the brain tissue of rats administered with L-GBCAs. To date, no effort has been made to analyze the Gd retained in the brain tissue fraction insoluble with mild buffer, because of the lack of methods able to solubilize it without compromising the nature of the contained Gd compounds. The speciation of the to-date unaccounted for Gd species remains therefore unknown. It is unclear whether GBCA is retained in the pellet as intact GBCA or whether it had undergone dissociation. In contrast to L-GBCAs, the presence of the kinetically very stable M-GBCAs in the pellet was hypothesized to be due to incomplete washout of entrapped GBCA inside cell debris. However, the fact that 3 washings of the remaining pellet were insufficient suggests that the mechanism of the Gd retention can be more complex.¹¹

The second drawback of the to-date reported procedures is the species-dependent nonquantitative recovery of Gd from the chromatographic column. The stationary phase is not entirely inert and may react with the Gd species depending on their stability. This concerns not only the GBCAs but also the species potentially present in the tissue, and

specially Gd³⁺. The mass balance of the introduced and recovered Gd has seldom been reported.²¹ To the best of our knowledge, the recoveries of Gd species from the SEC column have never been reported.^{11,20}

Finally, even if some evidence of the presence of Gd complexes with macromolecules in water-soluble fractions exists, the identity of this species remains unknown.

The aims of this study were to develop a method able to solubilize the brain tissue completely and to obtain an insight into the speciation of the additionally recovered Gd, as a function of GBCA. Rats administered with 3 GBCAs (1 macrocyclic and ionic GBCA gadoterate meglumine and 2 linear [1 ionic gadobenate dimeglumine and 1 non-ionic gadodiamide]) were investigated. Size exclusion chromatography was revisited with an emphasis of the compound-dependent column recovery of Gd and insight into the identity of the observed Gd species.

Materials and Methods

All animal experiments were conducted in full compliance with the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes.

Analytical reagent grade chemicals were used. They were obtained from Merck (Darmstadt, Germany) unless stated otherwise. Dionized water (18 MΩ cm) was obtained from a Milli-Q water purification system (Merck, Darmstadt, Germany).

Animal Model and Gadolinium-Based Contrast Agent Administration

To cover the different GBCA classes available on the market, 3 compounds were selected: 1 macrocyclic and ionic GBCA (gadoterate meglumine, Dotarem; Guerbet, Villepinte, France) and 2 linear—1 ionic (gadobenate dimeglumine, MultiHance; Bracco, Milan, Italy) and 1 nonionic (gadodiamide, Omniscan; GE Healthcare, Boston, MA). These GBCAs were used in the form of the commercially available solution for injection (0.5 M solutions). Four groups of healthy female Sprague Dawley rats (SPF/OFA rats; Charles River, L'Arbresle, France) randomly received an intravenous injection of 2.4 mmol/kg of GBCA each week during 5 consecutive weeks (cumulated dose of 12 mmol/kg) or no injection (control group). A total of 2.4 mmol/kg corresponds to 0.4 mmol/kg in humans according to the Food and Drug Administration guidance, after adjustment to the body surface area of the rat.²² Intravenous injections were performed in 1 tail vein under isoflurane anesthesia (IsoFlo; Axience, Pantin, France). The animals were sacrificed 1 week (W1) after the last injection. The GBCA dose and the sacrifice time were chosen as a compromise between the analysis of the residual Gd species and the sufficient Gd concentration for the sample preparation method optimization. At the completion of the washout period, the rats were euthanized under isoflurane anesthesia by exsanguination. The brains were removed and dissected to sample a half cerebellum, half cortex, and half subcortical brain. The cerebral tissues were stored at –80°C before sample preparation.

Sample Preparation

The cerebral tissue was homogenized using an Ultra-Turrax disperser (IKA-Werke, Staufen, Germany) in 100 mM ammonium acetate (pH 7.4; dilution factor of 6) in an ice bath. Then, the homogenate was divided into 400 μL or 650 μL aliquots and stored at –20°C.

The sequential extraction procedure, schematically presented in Figure 1, was based on the following:

1. Water extraction: The tissue homogenate was centrifuged using a Centrifuge 5804 R model (Eppendorf, Hamburg, Germany) for 30 minutes at 20,800g at 4°C. The water-soluble fraction was separated from the water-insoluble fraction and stored in –20°C. The water-insoluble fraction was washed with 0.5 mL of 100 mM ammonium acetate.

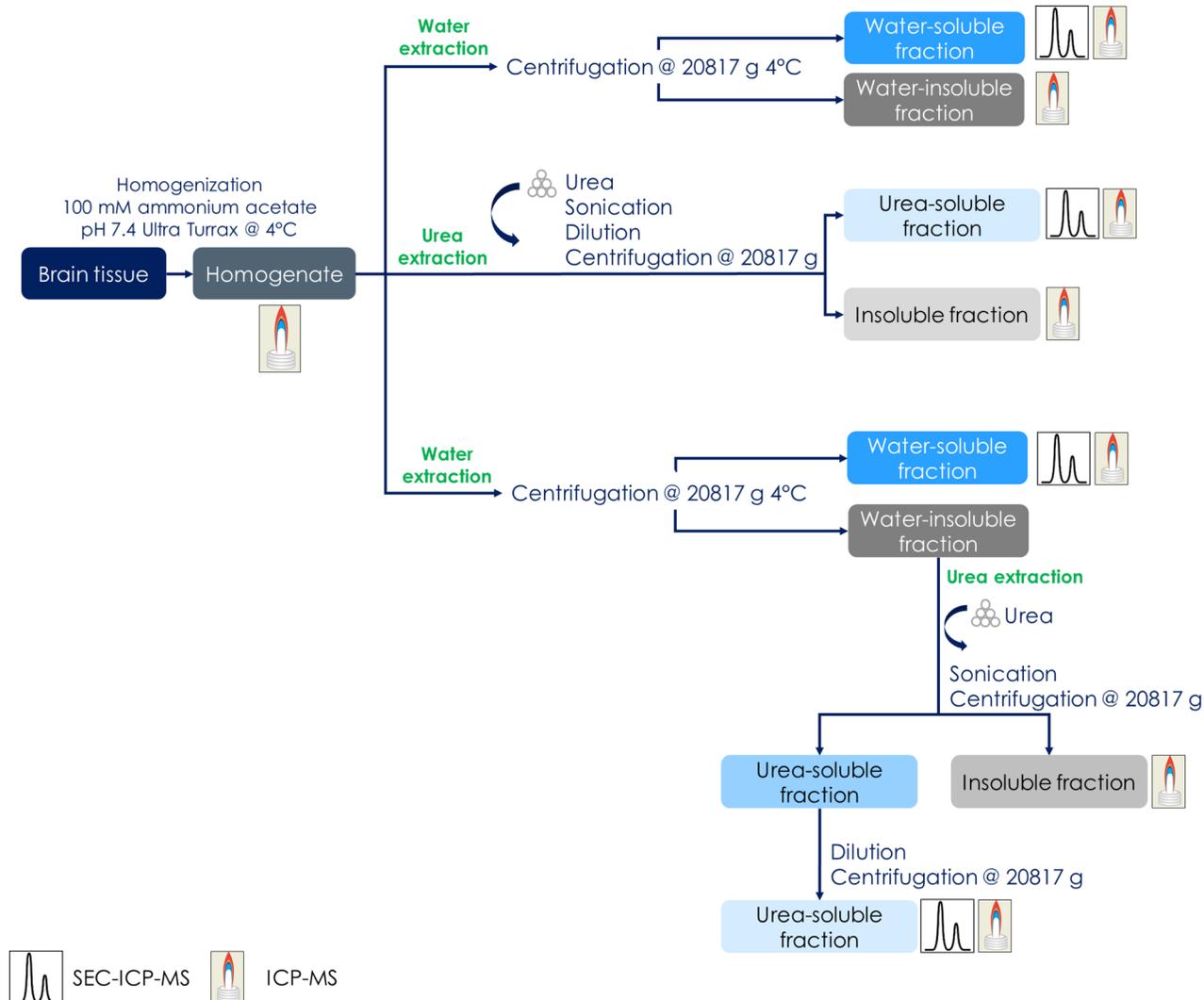


FIGURE 1. Two sample preparation procedures and the resulting fractions. Analytical methods corresponding to each fraction are shown schematically.

2. Urea extraction: The water-insoluble fraction was incubated in 1 mL of 5.6-M urea and sonicated using an ultrasonic liquid processor (Vibra-Cell; Sonics & Materials, Inc, Newtown, CT) in an ice bath. The urea-soluble fraction was collected and stored at 4°C. This fraction was twice diluted with 100 mM ammonium acetate (pH 7.4) and centrifuged before SEC analysis. The final pellet (urea-insoluble fraction) was washed 3 times with water to remove the remaining urea.

To compare the water and urea extractions, the urea was also applied directly to the cortex homogenate as presented in Figure 1.

Compound Stability

To control the stability of the GBCAs during the sample preparation procedure, cortex samples from untreated rats were spiked before the homogenization step with the investigated GBCAs to obtain a Gd concentration of 1 nmol/g. The spiked tissues were then treated in the same manner as the samples from the animals treated with GBCAs.

Total Gadolinium Determination

Samples of tissue homogenate (100 μ L) were digested on a hot plate with a mixture (3:1, vol/vol) of nitric acid and hydrogen peroxide

(400 μ L) at 80°C for 8 hours followed by dilution with water. In this last step, a standard solution of indium was added as an internal standard to obtain 1 ng/mL final concentration. Total Gd concentrations were measured in the homogenate and the supernatant with ICP-MS using an Agilent Model 7700x ICP-MS (Agilent Technologies, Santa Clara, CA). The ICP-MS instrument used for total Gd determination was optimized daily using a multielemental solution. A standard curve of inorganic Gd in 4% HNO₃ was constructed by recording the response of the ¹⁵⁸Gd isotope. The quantification range of the method was 0.010 to 1.0 ng/mL in solution. The Gd concentration in each fraction of cerebral tissue (homogenate and supernatant) was expressed as nmol/g and was calculated by multiplying the Gd concentration measured in the final solution by the dilution factor applied during sample preparation. The lower limit of quantification (LLOQ) of Gd was 0.02 nmol Gd/g in wet tissue complying with the criterion that the analyte response at the LLOQ should be at least 5 times the analyte response of the zero calibrator.

Data are expressed as mean \pm standard deviation (SD). Because of the limited number of biological replicates used for the Gd determination in most of the brain compartments ($n = 2$), the statistical analysis of obtained data was not performed.

Extraction Efficiency Measurement

To evaluate the amount of Gd extracted from the cerebral tissue to the soluble fraction(s), the extraction efficiency (*EE*) was calculated using the following equation: $EE = \frac{n_{Gd\ supernatant}}{n_{Gd\ homogenate}} \cdot 100\%$, where n_{Gd} is the number of moles of Gd. For sequential extraction, the amount of Gd in 2 water-soluble supernatants (first extraction and washing step) and in the 2.8-M urea-soluble fraction was considered.

Solubilization of Cerebral Tissue

The percentage of solubilization of a cerebral tissue (*BS*) was estimated using the following equation: $BS (\%) = \frac{m_{dry\ homogenate} - m_{dry\ pellet}}{m_{dry\ homogenate}} \cdot 100\%$. For this purpose, all the pellets were washed twice with 1 mL of water, lyophilized and weighed ($m_{dry\ pellet}$). The mass of dry homogenate was calculated using the following equation: $m_{dry\ homogenate} = m_{wet\ homogenate} \cdot f_{dry\ tissue}$, where the proportion of a dry tissue in a wet homogenate $f_{dry\ tissue}$ was estimated for 6 representative homogenates, which were lyophilized: $f_{dry\ tissue} = \frac{m_{dry\ homogenate}}{m_{wet\ homogenate}}$.

Enrichment Factor

The enrichment factor (EF) is a widely used metric for determining the number of times the analyte concentration has increased relative to its concentration without enrichment. The water, urea, and sequential extraction procedures were compared in terms of the EFs for Gd contained in the final insoluble residue. The EF was calculated as the ratio of the initial dry mass of the homogenate and the reduced final dry mass of the insoluble residue.

Gadolinium Speciation Analysis

The SEC-ICP-MS analysis was carried out using an high performance liquid chromatography 1260 system (Agilent Technologies) coupled to ICP-MS 7700x (Agilent Technologies). Gadolinium species were isocratically eluted either from a Superdex 75 or a Superdex 200 column (300 × 10 mm, GE Healthcare) with 100 mM ammonium acetate (pH 7.4) over 45 minutes at a flow rate of 0.7 mL/min. Because of the limited separation range of the Superdex 75 column (3–80 kDa), the Superdex 200 (separation range, 10–660 kDa) column was used to better estimate the molecular weight of the detected Gd species. The injection volume was 100 µL. The LLOQ of SEC-ICP-MS was estimated using standard GBCA solutions and set at 0.32 pmol/mL for all 3 GBCAs using the signal-to-noise > 5 criterion. To control the background level of Gd, the supernatants obtained from the samples of the control group were analyzed systematically at the beginning and at the end of the sequence as the blanks. The average blank chromatogram

per run was calculated and subtracted from the chromatograms unless specified otherwise.

The column recovery was verified for Gd in spiked samples from untreated animals and in the real samples from treated animals, comparing the total area of the peaks detected using the chromatographic column with the area of the peak obtained in flow injection mode (without column).

RESULTS

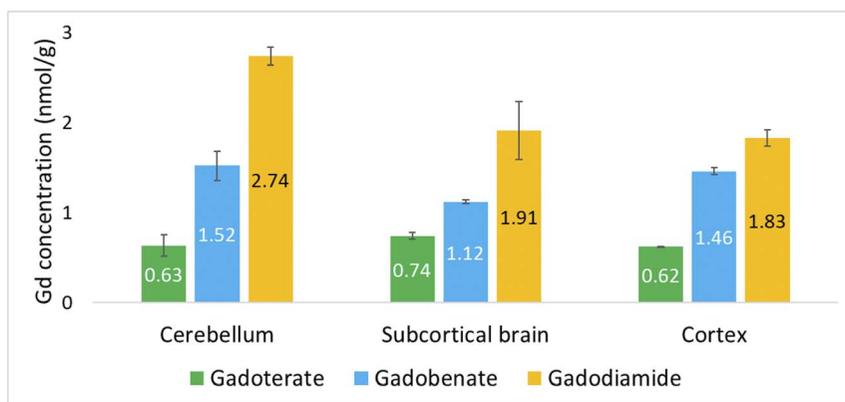
Total Gadolinium Determination

The total Gd concentrations determined are summarized in Figure 2. The concentration in the cerebellum obtained after injections of rats with gadoterate (0.63 ± 0.12 nmol/g) was lower than that obtained after injections with either linear contrast agents: gadobenate (1.52 ± 0.16 nmol/g) and gadodiamide (2.74 ± 0.10 nmol/g). In addition, the Gd concentration after injection of gadobenate was lower than that measured after injection of gadodiamide. The same trend was found for the subcortical brain and the cortex tissues.

Brain Solubilization and Extraction Recoveries

Table 1 summarizes the percentage of the brain solubilization, the Gd extraction efficiency data, and the EFs obtained using water, urea, and the sequential extraction for the samples from the treated groups. The extraction with the ammonium acetate buffer (water extraction) allowed the recovery of 68% to 72% of Gd initially present in the tissue for macrocyclic gadoterate. For L-GBCAs, the extracted amounts of Gd were much lower: 13% to 16% for gadobenate and 12% to 13% for gadodiamide. Water extraction managed to solubilize only 33% of the brain tissue matrix components. The cortex was better solubilized (65%–72%) using a chaotropic agent urea. It also resulted in the highest Gd extraction efficiencies of 47% ± 2% and 44% ± 7% for gadobenate and gadodiamide, respectively. The extraction for macrocyclic gadoterate was also improved (88% ± 3%).

The combination of water and urea extraction maximized the solubilization to reach 97% of brain tissue. Washing of the water-insoluble fraction allowed the recovery of an additional amount of Gd, similar for both brain structures, 12% to 15% for gadoterate but only 2% to 3% for gadobenate and gadodiamide (data not shown). In the second step, for gadoterate-containing samples, 15% to 19% of original Gd were extracted to urea-soluble fraction regardless of the brain matrix; consequently, almost no Gd (1%–2%) was found in the final, insoluble residue. In total, similar amounts of Gd (97% ± 1% and 102% ± 3%) were extracted from the cortex and cerebellum for macrocyclic



The Gd concentrations in the brain tissues were measured for n=2 biological replicates.

FIGURE 2. Accumulation of gadolinium (Gd) in the cerebellum, subcortical brain, and cortex 1 week after the fifth injection of gadolinium-based contrast agents.

TABLE 1. Brain Solubilization and Gadolinium Extraction Efficiencies in the Different Analytical Protocols Developed

Extractant	Brain Structure	Brain Solubilization (%)	Extraction Efficiency (%)			Enrichment Factor
			Gadoterate	Gadobenate	Gadodiamide	
Water	Cortex	33 ± 2	68 ± 2	13 ± 7	13 ± 1	1.4 ± 0.1
Urea		72 ± 2	88 ± 3	47 ± 2	44 ± 7	3.5 ± 0.2
Water + urea	Cerebellum	97 ± 1	102 ± 0	49 ± 1	48 ± 2	28 ± 7
Water		33 ± 2*	72 ± 1	16 ± 3	12 ± 1	
Water + urea		96 ± 1	97 ± 1	46 ± 4	34 ± 1	34 ± 14

Brain solubilization was calculated based on the different number of data points for each analytical procedure, for cortex samples: water extraction $n = 8$, urea extraction $n = 6$, and water + urea extraction $n = 6$; for cerebellum: water + urea extraction $n = 7$. Extraction efficiency was calculated based on $n = 2$ data points except water extraction for cerebellum $n = 5$ (gadobenate $n = 4$).

*Brain solubilization after water extraction was determined only for cortex; no significant differences between cortex and cerebellum were expected.

gadoterate. For gadobenate and gadodiamide, the total amount of Gd recovered from the cortex was $49\% \pm 1\%$ and $48\% \pm 2\%$ and from the cerebellum was $46\% \pm 4\%$ and $34\% \pm 1\%$, respectively. In addition to a lower recovery in comparison with the macrocyclic gadoterate, 1 significant difference was observed between the cortex and the cerebellum tissues for these L-GBCAs. Dilution of the 5.6-M urea-soluble fraction with ammonium acetate buffer (pH 7.4) decreased the amount of Gd for the cerebellum but not for cortex samples. Indeed, approximately half of the amount of Gd species extracted from the water-insoluble fraction to 5.6-M urea fraction was not soluble in 2.8-M urea (data not shown).

Table S1 (Supplemental Digital Content, <http://links.lww.com/RLI/A617>) shows high extraction efficiencies calculated for the brain samples from the control group spiked with either gadoterate, gadobenate, or gadodiamide, which suggests that no significant degradation of GBCAs occurred during sample preparation. Indeed, 85% to 96% of total Gd was extracted for all 3 GBCAs with urea. Water extraction resulted in slightly less efficient extraction (64% to 95%).

The Gd contained in the final, insoluble residue was enriched the most efficiently using sequential extraction procedure (EF of 28 ± 7 and 34 ± 14 for the cortex and cerebellum, respectively) compared with water (1.4 ± 0.1) and urea (3.5 ± 0.2) extractions.

Speciation of Gadolinium Using SEC-ICP-MS

Representative SEC-ICP-MS and SEC-UV chromatograms (Superdex 75 column) of the water- and urea-soluble fractions from the treated rat group are shown in Figure 3. The chromatograms for the brain samples from the control group spiked with 1 of the GBCAs are shown in Figure S1 (Supplemental Digital Content, <http://links.lww.com/RLI/A617>). A different number of Gd species was detected for linear and macrocyclic GBCAs.

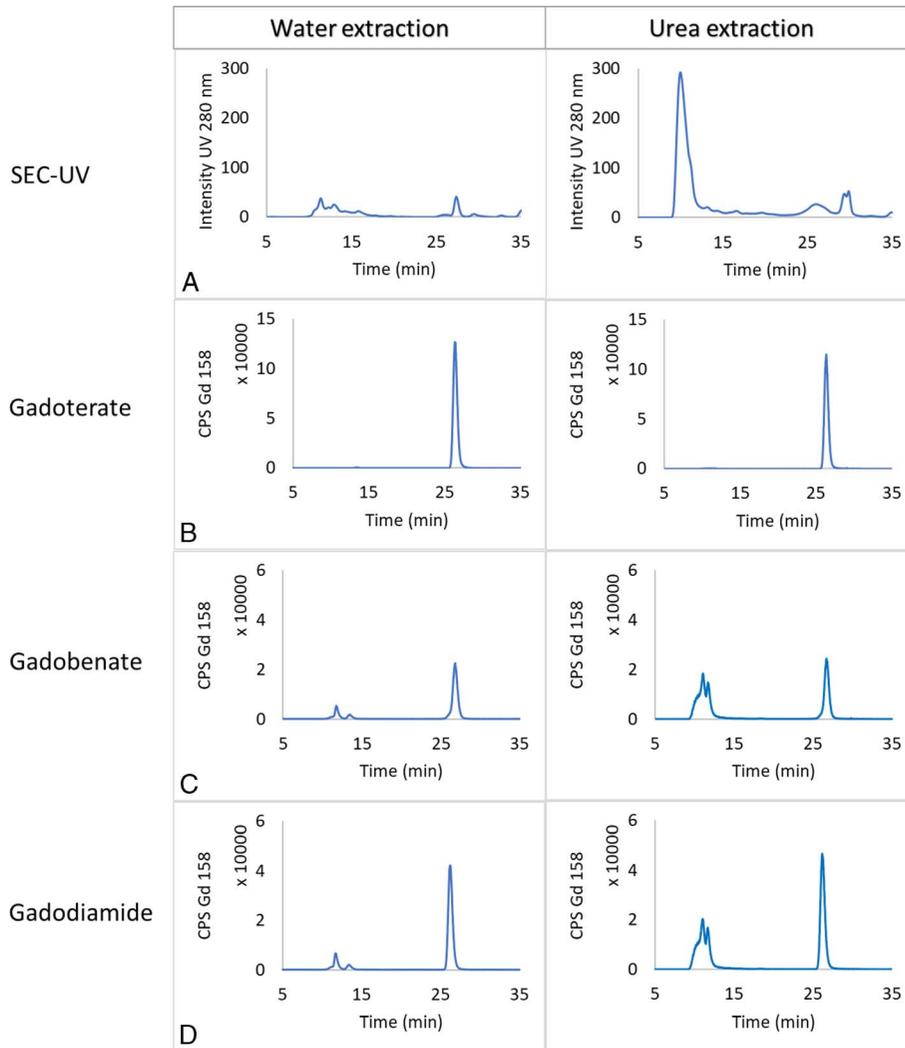
Water Extraction

For macrocyclic gadoterate, Gd was exclusively detected as 1 large peak with a similar retention time as the intact GBCA (RT, 26.3 minutes). On the other hand, for L-GBCAs, 3 distinct peaks were present at the retention times of $RT_1 = 11.7$ minutes, $RT_2 = 13.4$ minutes, and $RT_3 = 26.2$ minutes (for gadodiamide) or $RT_3 = 26.8$ minutes (for gadobenate). According to the column calibration, which is shown in Figure S2 (Supplemental Digital Content, <http://links.lww.com/RLI/A617>), the Gd species eluting at 11.7 minutes should be larger than 81 kDa, the 1 eluting at 13.4 minutes should be of approximately 66 kDa, and the last one, eluting between 20.1 and 29.3 minutes, should be smaller than 12 kDa. Therefore, the signal observed approximately 26 to 27 minutes corresponds to LMW molecules, such as the intact GBCA, whereas the peaks at 11.7 minutes and 13.4 minutes correspond

to HMW Gd species with the size of approximately of 66 kDa or larger. The column recoveries obtained for the water-soluble fraction of the control group samples spiked with standard GBCAs solutions and from the samples of treated rats are presented in Figure 4. The column recoveries for the spiked samples confirmed that intact GBCAs are eluted completely from the chromatographic column regardless of their stability with no degradation of GBCAs in the chromatographic conditions used for the analysis. Likewise, the column recovery obtained for the real samples after administration of gadoterate was $111\% \pm 2\%$, which proved that all Gd extracted to the water-soluble fraction of the brain was present solely as intact GBCA form. However, for the L-GBCAs gadodiamide and gadobenate, the column recovery was approximately $82\% \pm 2\%$, suggesting that a no negligible amount of Gd present in the water-soluble fraction was retained on the stationary phase of the chromatographic column, which would happen, for instance, for Gd present as weakly bound Gd^{3+} with endogenous ligands present in the sample.

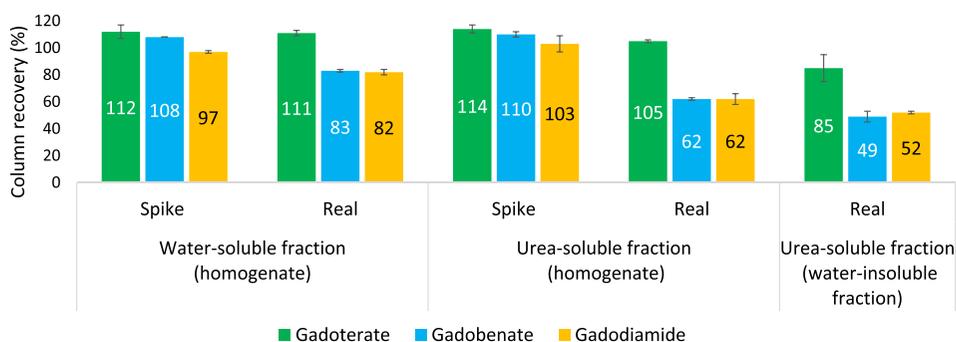
Urea Extraction

No change in the peak intensity was observed for the samples from the control group spiked with either gadoterate, gadobenate, or gadodiamide (see Fig. S1, Supplemental Digital Content, <http://links.lww.com/RLI/A617>). Moreover, the examination of column recovery showed complete elution of Gd from the chromatographic column (103%–114%) for all 3 GBCAs, proving their stability in these conditions. A representative SEC-ICP-MS chromatogram of the supernatant (cortex W1) obtained for gadoterate sample (Fig. 3B) shows that similarly to water extraction Gd was eluted completely as 1 peak at the retention time $RT_1 = 26.4$ minutes. Typical SEC-ICP-MS chromatograms of the urea-soluble fraction of cortex (W1) for gadobenate and gadodiamide are presented in Figures 3C and D. At least 3 Gd peaks were observed for these L-GBCAs. Two of the peaks were eluted very close to the void volume of Superdex 75 column in the HMW elution range ($RT_1 = 11.1$ minutes, $RT_2 = 11.7$ minutes), whereas 1 peak was observed in the LMW range at 26.7 minutes. The 2 peaks in the HMW fraction were not well separated, and there may be another broad peak of smaller intensity, eluting in the void volume of the column (10.3 minutes) but covered by the peak at 11.1 minutes. Because of the low resolution and the limited separation range of the chromatographic column used for this analysis, the hydrodynamic volume of the Gd species present in the HMW fraction could only be estimated as larger than 80 kDa. Also, the Gd species detected by SEC-ICP-MS did not account for all the Gd present in the supernatant. The column recoveries for both L-GBCAs showed that 38% of extracted Gd was retained on the chromatographic column strongly, suggesting the presence of other Gd species (see Fig. 4).



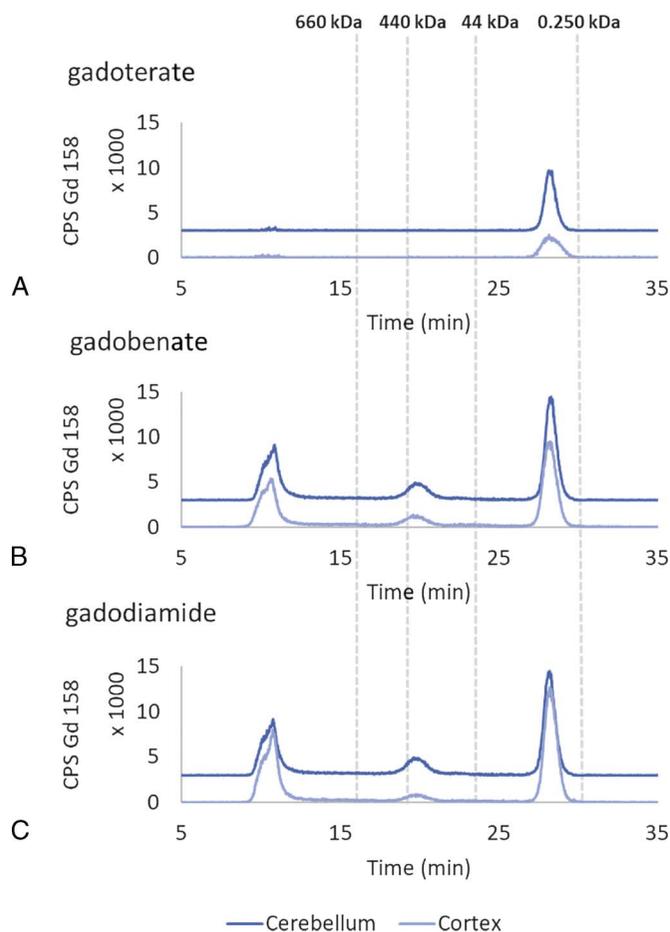
The chromatograms after urea extraction are shown without blank subtraction. The chromatograms after water extraction were corrected by the dilution factor used for the urea-based protocol (determined experimentally).

FIGURE 3. Molecular size distribution of Gd species recovered to water- and urea-soluble fractions from the cortex homogenate. Typical size exclusion chromatography UV (A) and size exclusion chromatography–inductively coupled plasma–mass spectrometry (B–D) chromatograms obtained using Superdex 75 column.



Data points for each group are n=2.

FIGURE 4. Gd recovery from the Superdex 75 and 200 columns. Data obtained for the samples from the untreated animals spiked with gadolinium-based contrast agent standards and from the treated animals. Column recoveries determined for Gd species present in the water- and urea-soluble cortex fractions after extraction from either the homogenate or the water-insoluble fraction.



The data was obtained using Superdex 200. To avoid overlapping of the chromatograms, the offset of 3000 CPS was added to the cerebellum series.

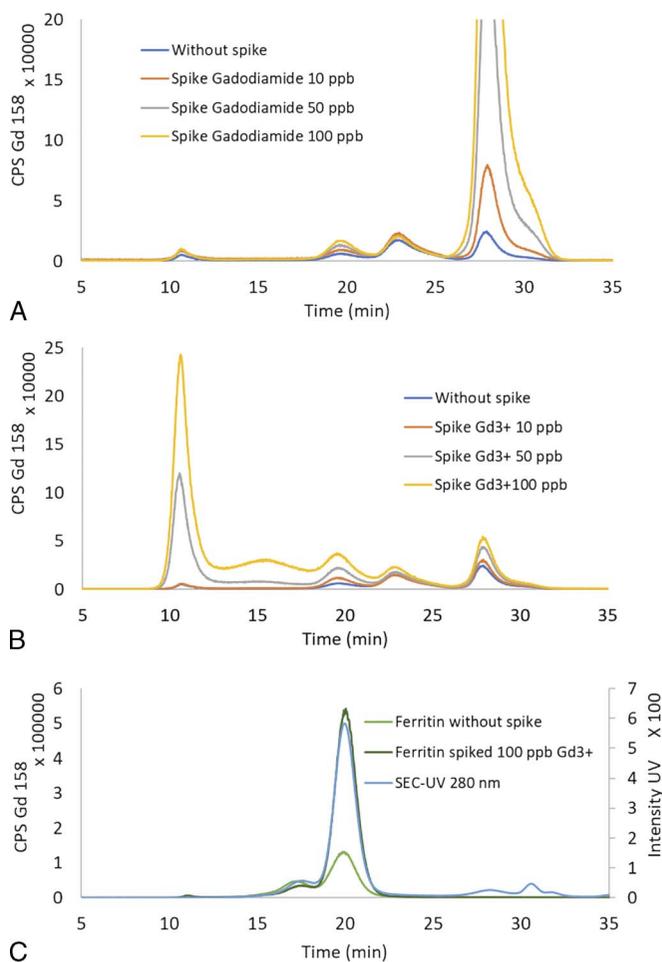
FIGURE 5. Molecular size distribution of Gd species recovered to 2.8-M urea-soluble fraction from the cortex and cerebellum water-insoluble fractions.

Sequential Extraction

Representative SEC-ICP-MS chromatograms obtained using Superdex 200 column of the 2.8-M urea-soluble fractions extracted from the water-insoluble fractions are shown in Figure 5. For gadoterate, only 1 species was detected in the LMW fraction at $RT_1 = 28.1$ minutes. For L-GBCAs, 3 Gd peaks were observed at the following retention times: $RT_1 = 11.0$ minutes, $RT_2 = 20.1$ minutes, and $RT_3 = 28.3$ minutes, which, according to the calibration of the Superdex 200 column (Fig. S3, Supplemental Digital Content, <http://links.lww.com/RLI/A617>), corresponds to the MW_1 larger than 660 kDa, MW_2 of approximately 440 kDa, and MW_3 inferior to 44 kDa. The column recoveries of water-soluble fractions are in good agreement with previously shown data (gadoterate $111\% \pm 2\%$, gadobenate $83\% \pm 1\%$, and gadodiamide $82\% \pm 2\%$) and are shown in Figure S4 (Supplemental Digital Content, <http://links.lww.com/RLI/A617>) together with the data obtained for the cerebellum samples. The column recoveries obtained for 2.8-M urea-soluble cortex fractions are presented in Figure 4. Regarding the urea-soluble fractions, quantitative elution of Gd from the chromatographic column ($85\%–98\%$) was observed only for gadoterate. For gadobenate and gadodiamide, half of the extracted Gd was retained on the stationary phase for cortex and 60% for cerebellum samples.

Insight Into the Nature of the Extracted Gadolinium Ligand Species

Figures 6A and B demonstrate SEC-ICP-MS chromatograms of water-soluble fraction of cortex sample (W1) from gadodiamide group spiked with 10, 50, or 100 ppb of either Gd^{3+} or gadodiamide standards. The chromatograms of the sample spiked with a gadodiamide standard showed that only the intensity of the peak eluting at 27.9 minutes increased. In contrast, when the same sample was spiked with Gd^{3+} , the intensity of the peak eluting at 10.7 minutes increased significantly, and a slight increase in the height of 2 peaks eluting at 19.6 and 27.9 minutes was observed. Therefore, only the ionic Gd but not the GBCA showed a strong affinity toward the HMW ligands. Figure 6C shows SEC-UV-ICP-MS chromatograms obtained for a ferritin standard solution before and after spiking with ionic Gd to the final Gd concentration of 100 ppb. Two different peaks are present at the retention times: $RT_1 = 17.2$ minutes and $RT_2 = 20.1$ minutes, proving that ferritin bound some ionic Gd both from the chromatographic column and from the spike.



The data was obtained using Superdex 200 column.

FIGURE 6. Insight into the nature of the extracted Gd species. Size exclusion chromatography–inductively coupled plasma–mass spectrometry chromatograms of water-soluble fraction of cortex samples of gadodiamide spiked with either 10, 50, or 100 ppb of gadodiamide (A) or ionic gadolinium (B). Size exclusion chromatography–inductively coupled plasma–mass spectrometry of ferritin standard alone and spiked with 100 ppb of ionic gadolinium (C).

DISCUSSION

Total Gadolinium Concentrations

The total Gd concentrations measured by ICP-MS in the cerebellum, subcortical brain, and in the cortex confirmed the class effect resulting in the higher Gd concentrations accumulated after L-GBCAs administrations.¹⁶

Extraction Efficiency

Water Extraction

In the previous bioanalytical studies, water extraction providing mild conditions during sample processing (absence of detergents, chaotropic agents, and organic solvents) combined with pH close to the physiological value (7.4) was typically used to minimize the risk of changes of the original Gd speciation. Our data obtained for the samples from the animals treated with GBCAs confirmed that this extraction approach failed to recover all the Gd to the water-soluble fraction from the tissue, which was further analyzed by high performance liquid chromatography-ICP-MS. These results are consistent with previous findings, although the availability of precisely calculated extraction efficiency values for the brain tissue is limited in the literature.^{11,20} In this study, 68% to 72% of Gd from the macrocyclic gadoterate and only 12% to 16% from the linear gadobenate and gadodiamide were found in the water-soluble brain fraction. Such low recoveries limit significantly the investigation of Gd species by hyphenated techniques and leave unanswered the question of the nature of Gd species present in the pellet after administration of GBCAs. Indeed, it should be determined whether Gd remained in the water-insoluble fraction because of the unsuitable extraction conditions or rather because of the insoluble character of Gd species. In the case of L-GBCAs, the most likely explanation is that, because of their lower kinetic stability, they gradually dissociate in tissues and form nonextractable in water Gd species, such as, for example, inorganic salts or strong complexes with biomolecules forming the pellet. Interestingly, Kahakachchi et al²³ reported low extraction efficiency (5%) similar to L-GBCAs from this study (8%–18%) when bovine muscle powder was spiked with ionic Gd³⁺ and extracted with water. Moreover, the insoluble deposits of Gd associated with phosphorus were observed using TEM-Energy-dispersive X-ray spectroscopy in skin tissues and using TEM and nanoSIMS in the cerebellum.¹⁹ However, it is unlikely that Gd is present in the pellet only in solid binary forms such as GdPO₄, Gd₂(CO₃)₃, and Gd(OH)₃ because it could not generate the high signal intensity observed on T1-weighted magnetic resonance images. Indeed, the relaxivity values (60 MHz, 37°C) measured for Gd phosphate, carbonate, and hydroxide in water and in skin samples are very low (0.01–0.29 mM⁻¹ s⁻¹).²⁴

Optimization of Brain Solubilization and Extraction Conditions

Water extraction was found to solubilize only 33% of brain tissue, which may explain the poor extraction recoveries of Gd species. Therefore, it was decided to develop a more efficient brain tissue solubilization procedure. The brain is composed of approximately 77% to 78% of water, 10% to 12% of lipids, 8% of proteins, 1% of carbohydrates, 2% of soluble organic substances, and 1% of inorganic salts.²⁵ A dedicated approach based on the use of chaotropic agent urea was optimized to assure its solubilization in conditions preserving the analyte species. Indeed, urea appeared to have higher capacity to solubilize the brain tissue than water, allowing the dissolution of 72% of the cortex matrix while preserving the initially injected form of GBCAs (see Fig. S1, Supplemental Digital Content, <http://links.lww.com/RLI/A617>). More importantly, it improved Gd extraction. Almost half of Gd was recovered from the brain tissue for L-GBCAs (47% ± 2% and 44% ± 7% for gadobenate and gadodiamide, respectively). Nevertheless, approximately half of total Gd remained in the insoluble residue. It is

consistent with the imaging experiments showing the electron-dense deposits containing Gd in the brain.^{8,19} Indeed, the solid forms cannot be extracted using the procedures developed in this work. In the case of macrocyclic gadoterate, all the Gd was extracted using urea.

Total Brain Solubilization: The Sequential Extraction

To solubilize brain tissue completely, a combination of water and urea extraction was applied in sequence. For each step of this solubilization process, the extraction efficiency was calculated showing that in total 97% to 102% for gadoterate and 34% to 49% of Gd species for L-GBCAs could be solubilized in the conditions guaranteeing the stability of initially injected GBCA form. Note that sequential extraction did not maximize the extraction efficiency in comparison to urea extraction alone, even if it solubilized almost completely the brain tissue. However, by reducing the mass of the final insoluble residue, the concentration of Gd species in that fraction was considerably increased compared with the initial concentration in the homogenate. Indeed, the Gd EFs in the insoluble fraction reached 28 ± 7 and 34 ± 14 for the cortex and cerebellum, respectively, which might be an important improvement for future studies of these insoluble Gd species.

Speciation of Gadolinium in the Water-Soluble Part of Brain Tissue

In the case of macrocyclic gadoterate, the extracted Gd was detected exclusively as the intact GBCA in water-soluble brain fraction. For the L-GBCAs, apart from the dominating intact GBCA form, the tiny amount of Gd bound to macromolecules was observed as already reported by Frenzel et al¹¹ and Robert et al.²⁰ Water allows the extraction of highly soluble Gd species such as intact GBCA or macromolecules that can be proteins, carbohydrates, or some fragments of more complex endogenous molecules. However, based on the determination of the column recovery, approximately 18% ± 2% of Gd was present in the water-soluble fraction in other than previously reported form(s) that was retained on the stationary phase (see Fig. 4).

Speciation of Gadolinium in the Urea-Soluble Part of Brain Tissue

All Gd was extracted from the brain homogenate of rats treated with gadoterate using urea procedure, and SEC-ICP-MS demonstrated that it was present exclusively as intact GBCA form in the cortex (Fig. 3B). This result confirmed that, in the case of M-GBCA, partial loss of Gd in the water-insoluble fraction was not due to the formation of nonextractable Gd species but rather due to mechanical capture of GBCA form in tissue or during sample processing. This part cannot be totally extracted with water, but it is rapidly released when the tissue is better solubilized with urea. The Gd extraction was also improved for the L-GBCAs. Interestingly, 2 forms of Gd were better extracted in urea compared with water-soluble fraction: Gd bound to macromolecules and weak complexes of Gd³⁺ with endogenous ligands, in which presence was deduced from the incomplete elution of Gd from the chromatographic column (see Fig. 4). Indeed, the column recoveries were 82% and 62% in water- and urea-soluble fractions, respectively. The HMW Gd species detected in the urea-soluble fraction were approximately 5-fold more abundant than those obtained after water extraction. At high concentrations, urea is commonly used to solubilize more hydrophobic, membrane-associated protein, and also proteoglycans,²⁶ by breaking the intermolecular and intramolecular noncovalent interactions (such as hydrogen bonds, dipole-dipole, and hydrophobic interactions). Approximately 50% of brain proteins are insoluble in water, acid, and alkaline²⁵; thus, it is possible that urea allowed to solubilize some of them without disrupting their interaction with Gd. The SEC-UV chromatograms obtained for water- and urea-soluble fractions (cf Fig. 3A) support this hypothesis. Indeed, 1 relatively intense peak was detected using a UV detector at 280 nm at RT₁ = 11.2 minutes in urea-soluble

fraction but was absent in the water one. This peak is eluted very close to or in the void volume of the column, which strongly suggests the presence of very large molecules or aggregates. Indeed, by breaking the secondary protein structure, urea increases the number of hydrophobic fragments exposed to the solvent, and thus the possibilities of protein aggregation.²⁷

Speciation of Gadolinium in the Water-Insoluble (Urea-Soluble) Part of Brain Tissue

The Gd extracted with urea from the water-insoluble brain fraction was demonstrated to be entirely present in the GBCA form in the gadoterate-treated rats. For L-GBCAs (gadobenate and gadodiamide), not only the intact GBCA form was detected but also 2 different Gd species in high-molecular fraction. To estimate more precisely the size of these Gd species, a column (Superdex 200) with a much larger separation range (10–660 kDa) than that of Superdex 75 was used. Consequently, the separation of Gd species eluting in the HMW fraction was improved (cf Fig. 5). Two different Gd species with molar masses of (1) approximately 440 kDa and (2) larger than 660 kDa were observed. The Gd bound to macromolecules of approximately 440 kDa is likely to correspond to the Gd species detected previously in the water-soluble fraction of the brain by Frenzel et al¹¹ and Robert et al²⁰ who reported sizes of 250 kDa or superior to 66 kDa, respectively. Our result obtained using the Superdex 200 column (440 kDa) fits better with the estimation of Frenzel et al¹¹ who used a similar column with a stationary phase made of cross-linked agarose and dextran. The Gd species with hydrodynamic size larger than 660 kDa were not detected previously in water-soluble fractions. Also, the presence of another, not yet defined, Gd species was detected by controlling the column recovery. Indeed, for L-GBCAs, the column recovery was complete neither in water nor in urea extracted fractions (see Fig. 4; Fig. S4, Supplemental Digital Content, <http://links.lww.com/RLI/A617>). As SEC is known for the incomplete recovery of ionic forms of metals or weak metal complexes,²⁸ our results suggest that a significant amount of Gd was present in the form of weak or in other terms labile complexes of Gd³⁺ with some endogenous molecules. The released Gd³⁺ is retained on the stationary phase, and the corresponding ligand elutes from the analytical system without being detected by ICP-MS. The identification of *in vivo* Gd³⁺ complexes with weakly bound ligands remains to be a challenge.

Insight Into the Nature of the Extracted Gadolinium Ligand Species

Another piece of argument in favor of the presence of HMW excess ligand strongly binding Gd³⁺ was demonstrated by the rapid complexation of ionic Gd³⁺ added to the water-soluble fraction. This finding, corroborating the transmetalation hypothesis, represents substantial progress toward a better understanding of the processes involved in the residual Gd presence in the brain and its putative biological consequences. It is also important to highlight that the interaction between Gd and macromolecules turned out to be rather strong because it was not disrupted by denaturing conditions (concentrated urea) combined with probe sonication. Consequently, the presence of relatively specific Gd binding sites, and thereby its affinity toward an endogenous molecule, might be hypothesized. Several potential Gd targets have been proposed such as Ca²⁺-binding proteins, apotransferrin,²⁹ ferritin,^{30,31} neuromelanins,³² polysialic acid,²¹ or proteoglycans³³ in the literature. Our attention was directed to the iron-storing protein ferritin, because we observed that ferritin was able to interact with Gd retained on the chromatographic column and also with Gd³⁺ added to ferritin solution (cf Fig. 6C). Ferritin is also known to bind *in vitro* and *in vivo* other metals, such as Cu, Zn, Cd, and also Tb.³⁴ Interestingly, the nonferrous metal ions can bind to the iron oxyhydroxide core, which is stored in the central cavity of the ferritin³⁵ that can include up to 4500 iron atoms with variable amounts of phosphate. Gadolinium's

high affinity to the phosphate ion is well known.³⁶ Recently, it was shown that nanomolar concentrations of Gd³⁺ ions bind to the ferritin mineral core.³⁰ In our study, the ferritin spiked with ionic Gd produced a ¹⁵⁸Gd peak at the same retention time as the HMW Gd species present both in water- and urea-soluble fractions. Figure S5 (Supplemental Digital Content, <http://links.lww.com/RLI/A617>) represents the chromatograms monitored not only for ¹⁵⁸Gd but also for ⁵⁴Fe confirming the coelution of the iron and Gd peak at the retention time of 20.1 minutes (440 kDa) in the urea-soluble fraction of the cerebellum. However, because of the low resolution of SEC, the unambiguous identification of the nature of these Gd-containing macromolecules based on the standard retention time matching is not sufficient. Unfortunately, considering the matrix complexity, the size of the molecule, and the low concentrations involved, alternative techniques to corroborate the proposed identity of the complex seem to be lacking. Nevertheless, the recent *in vitro* study demonstrated the capacity of the Gd³⁺-ferritin nanoparticles to shorten the proton relaxation times.³⁰ Taken together, it is very likely that ferritin is 1 of the macromolecules interacting with dissociated Gd³⁺ and is responsible for the T1 signal intensity enhancement in iron-rich brain structures observed in patients after repeated administrations with L-GBCAs.

Conclusions

The combination of water and urea extraction allowed almost complete solubilization of the brain tissue and gave a valuable insight into the identity of the Gd species, 1 week after the last injection that had so far escaped the analysis. The macrocyclic gadoterate was found to be accumulated exclusively as intact in the brain. For both L-GBCAs gadobenate and gadodiamide, it was demonstrated that the amount of Gd bound to macromolecules and other endogenous ligands have been largely underestimated in the literature. Moreover, spiking experiments showed that the macromolecules present in the brain interacted only with the ionic Gd³⁺ rather than with the intact GBCA form, and the determination of the column recovery indicated the presence of labile complexes of Gd³⁺. Ferritin was identified as a potential endogenous macromolecule interacting with the dissociated Gd³⁺. Our results emphasize the validity of the dechelation hypothesis for L-GBCAs, such as gadobenate and gadodiamide, and the stability of a macrocyclic GBCA in the brain. For the linear GBCAs, the insoluble brain fraction composed of 3% of the initial tissue was found to contain more than 50% of Gd in unidentified form(s). Further investigations of these species would be interesting for a thorough evaluation of their potential long-term impact.

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