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To cite this version:
Izabela Strzeminska, Cécile Factor, Philippe Robert, Anne-Laure Grindel, Pierre-Olivier Comby, et al.. Long-Term Evaluation of Gadolinium Retention in Rat Brain After Single Injection of a Clinically Relevant Dose of Gadolinium-Based Contrast Agents. Investigative Radiology, Lippincott, Williams & Wilkins, 2020, 55 (3), pp.138-143. 10.1097/RLI.0000000000000623. hal-02481979

HAL Id: hal-02481979
https://hal-univ-pau.archives-ouvertes.fr/hal-02481979
Submitted on 16 Nov 2020

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Long-Term Evaluation of Gadolinium Retention in Rat Brain After Single Injection of a Clinically Relevant Dose of Gadolinium-Based Contrast Agents

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Purpose: The aim of this study was to investigate the presence and chemical forms of residual gadolinium (Gd) in rat brain after a single dose of Gd-based contrast agent.

Methods: Four groups of healthy rats (2 sacrifice time-points, n = 10/group, 80 rats in total) were randomized to receive a single intravenous injection of 1 of the 3 Gd-based contrast agents (GBCAs) (gadodiamide, gadobenate dimeglumine, or gadoterate) at a clinically relevant dose (0.067 ± 0.013 nmol/g; P < 0.05). Gadolinium speciation analysis of the water-soluble fraction showed that, after injection of the macrocyclic gadoterate, Gd was still detected only in its intact, chelated form 5 months after injection. In contrast, after a single dose of linear GBCAs (gadobenate and gadodiamide), 2 different forms were detected: intact GBCA and Gd bound to soluble macromolecules (above 80 kDa). Elimination of the intact GBCA form was also observed between the first and fifth month, whereas the amount of Gd present in the macromolecular fraction remained constant 5 months after injection.

Conclusions: A single injection of a clinically relevant dose of GBCA is sufficient to investigate long-term Gd retention in the cerebellar parenchyma. Administration of linear GBCAs (gadodiamide and gadobenate) resulted in higher residual Gd concentrations than administration of the macrocyclic gadoterate. Speciation analysis of the water-soluble fraction of cerebellum confirmed washout of intact GBCA over time. The quantity of Gd bound to macromolecules, observed only with linear GBCAs, remained constant 5 months after injection and is likely to represent a permanent deposition.

Key Words: gadolinium-based contrast agent, cerebellum, gadolinium species, gadolinium retention, gadodiamide, gadobenate, gadoterate

Gadolinium (Gd)-based contrast agents (GBCAs) constitute a major research topic in the field of clinical magnetic resonance imaging (MRI). Since 2014, numerous clinical and preclinical studies have demonstrated increased signal intensity (SI) on unenhanced T1-weighted MRI images in specific structures of the healthy brain after repeated administrations of linear GBCAs.1–4 The quantitatively and qualitatively variable presence of Gd in cerebral parenchyma according to the molecular class of the GBCA was confirmed using inductively coupled plasma mass spectrometry (ICP-MS) and transmission electron microscopy–energy dispersive x-ray spectrometry.5,6 Gadolinium retention in the brain raises important safety concerns because of the acute and chronic toxicities of ionic Gd7–9 and its deleterious role in the pathophysiology of nephrogenic systemic fibrosis.6 Nephrogenic systemic fibrosis is an adverse reaction primarily related to administration of linear GBCAs in patients with severe renal disease.10

Two types of GBCAs are distinguished according to the chemical structure of the ligand associated with Gd: linear and macrocyclic GBCAs. An overview of various stability parameters of 3 GBCAs is presented in Table 1. Thermodynamic stability (which reflects the affinity of Gd for its ligand) and kinetic stability (which reflects the rate at which the equilibrium between the metal and its ligand is reached) are generally both used to describe the physicochemical characteristics of GBCAs.11 High kinetic stability provided by the macrocyclic structure minimize dechelation both in vitro and in vivo. Compared with macrocyclic GBCAs, linear chelates present moderate or poor kinetic stability with a higher likelihood of dechelation.11,12 Numerous clinical and preclinical studies have suggested that Gd retention in tissues is inversely correlated with the thermodynamic and kinetic stability of the GBCA injected.13–15 Thus, linear GBCAs result in more intense and more persistent Gd retention than macrocyclic GBCAs. Indeed, 2 studies of long-term elimination kinetics from the rat brain tissues showed rapid washout of Gd after repeated injections of macrocyclic GBCAs.14,16 Conversely large amount of Gd remained in the brain 1 year after the administration of the linear GBCAs. Gadolinium species present in the cerebellar parenchyma of rats have been studied by size-exclusion chromatography (SEC) coupled to ICP-MS. These studies also
TABLE 1. Overview of the Various Parameters Reflecting the Stability of GBCAs

| Trade Name | Generic Name | Type       | Ionic/Nonionic | Excess Ligand | Log K<sub>SM</sub><sup>‡</sup> pH = 7.4<sup>‡</sup> | Log K<sub>ML</sub> pH = 7.4<sup>‡</sup> | Half-Lives T<sub>1/2</sub> pH = 1<sup>‡</sup> | Half-Lives T<sub>1/2</sub> pH > 5<sup>‡</sup> |
|------------|--------------|------------|----------------|---------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Dotarem    | Gadoterate meglumine | Macrocyclic | Ionic          | No            | 25.6                        | 19.3                        | 26.4 h (37°C)               | 37 y (pH = 5.3, 25°C)       |
| MultiHance | Gadobenate dimeglumine | Linear | Ionic          | No            | 22.6                        | 18.4                        | <5 s (25°C)†                | 5–7 d (pH = 7.4, 25°C) C<sup>†</sup> |
| Omniscan   | Gadodiamide   | Linear     | Nonionic       | Cu-DTPA-BMA   | 16.9                        | 14.9                        | <5 s (25°C)†                | 5–7 d (pH = 7.4, 25°C) C<sup>†</sup> |

*The values of thermodynamic constants were taken from Port et al.10
†The values of half-lives were taken from Frenzel et al.,11 and were determined in absence of a biological matrix.
‡The same value was attributed for each linear GBCA, as the linear GBCA for which this parameter was determined was not specified.

MATERIALS AND METHODS

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

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FIGURE 1. Sample preparation process and resulting fractions. Analytical methods corresponding to each fraction are shown.

Total Gd Determination

Samples (either 100 μL or the whole pellet) were digested on a hot plate with a mixture (3:1, vol/vol) of nitric acid and hydrogen peroxide (either 400 μL or a volume calculated to respect the dilution factor of 5) at 80°C for 8 hours followed by dilution with water. In this last step, indium was added as internal standard to obtain 1 ng/mL final concentration. Total Gd concentrations were measured in a homogenate, a supernatant, and a pellet with ICP-MS using an Agilent Model 7500 or 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 4.2% HNO₃ was used by monitoring the response of the ¹⁵⁶Gd isotope. The quantification range of the method was 0.010 to 1.0 ng/mL in solution for the first time-point (M1) and 0.001 to 0.5 ng/mL for the second time-point (M5). The Gd concentration in each fraction of cerebellum (homogenate, supernatant, and pellet) was expressed as nanomole per gram and was calculated by multiplying the Gd concentration measured in the final solution by the dilution factor applied during sample preparation, including the homogenization step for calculation for the cerebellum. The corresponding lower limits of quantification (LLOQs) of Gd in the cerebellum were 0.018 nmol/g in wet tissue (M1) and 0.002 nmol/g in wet tissue (M5), complying with the criterion that the analyte response at the LLOQ should be at least 5 times the analyte response of the zero calibrator.

Gd Speciation Analysis in the Cerebellum

To investigate the presence of Gd species in the cerebellum after a single dose of GBCA, samples taken at the M1 and M5 time-points were analyzed by SEC-ICP-MS using the 1200 or 1260 HPLC separation system (Agilent) with detection by an ICP-MS Agilent Model 7700x. Gadolinium species were isocratically eluted from a Superdex-75 column (300 × 10 mm; GE Healthcare, Chalfont St Giles, United Kingdom) with 100 mM ammonium acetate (pH 7.4) over 45 minutes at a flow rate of 0.7 mL/min. The size-exclusion column was calibrated using a series of different molecular weight markers to correlate with the elution volume (retention time). The molecular weight markers were Blue Dextran, transferrin, bovine serum albumin, ovalbumin, carbonic anhydrase, cytochrome C, and Gly-Tyr. The LLOQ of SEC-ICP-MS was estimated using standard GBCA solutions and set at 0.318 pmol/mL for all 3 GBCAs for both time-points (M1 and M5), complying with the criterion that the signal-to-noise ratio must be at least 5. To control for the background level of Gd, the supernatants obtained from the samples of the control group were run between the samples of the GBCA group as blanks. The average blank chromatogram per run was calculated (6 runs in total) and subtracted from the chromatograms of the corresponding runs of the GBCA groups. To evaluate the amount of water-soluble Gd extracted from the cerebellum and analyzed by SEC-ICP-MS, the extraction efficiency was calculated using the following equation: extraction efficiency = \( \frac{n_{Gd\ supernatant} + n_{Gd\ pellet}}{n_{Gd\ homogenate}} \times 100\% \), where \( n_{Gd} \) is the number of moles of Gd. In addition, the mass balance was verified using the following equation: mass balance = \( \frac{n_{Gd\ homogenate} + n_{Gd\ pellet}}{n_{Gd\ supernatant}} \).

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Due to the nonhomogeneous variance of total Gd concentrations, and the nonnormal distribution of some groups (D’Agostino-Pearson test), the differences between these data were analyzed using a Kruskal-Wallis test followed by a Dunn test. For calculation of means and SDs, and for statistical analysis of total Gd concentration in the cerebellum, values less than LLOQ were arbitrarily replaced by the LLOQ value. Outliers for all data were detected and excluded from the analysis according to the ROUT method (Q = 1%). Statistical analyses were carried out using GraphPad Prism 7 software (GraphPad Software Inc, San Diego, CA). Differences were considered significant at \( P \leq 0.05 \).

RESULTS

Total Gd Determination

Total Gd concentrations in the cerebellum are shown in Figure 2. At the 2 time-points, total Gd concentrations for gadodiamide, the least stable GBCA, were significantly higher than the concentration measured for gadoterate (\( P < 0.0001 \)). Similarly, the highest Gd concentration was found for gadodiamide (M1: 0.280 ± 0.060 nmol/g; M5: 0.193 ± 0.023 nmol/g) followed by gadobenate dimeglumine (M1: 0.093 ± 0.020 nmol/g; M5: 0.067 ± 0.013 nmol/g). The lowest total Gd concentration was therefore detected for gadoterate meglumine (M1: 0.019 ± 0.004 nmol/g; M5: 0.004 ± 0.002 nmol/g). At both time-points, Gd concentrations after injection of gadobenate, a linear GBCA, were significantly lower (\( P < 0.05 \)) than those measured after injection of gadodiamide. At M5, the Gd concentration after injection of gadoterate was significantly lower than that measured after injection of gadobenate (\( P < 0.05 \)). A similar but nonsignificant trend was observed at M1, which could be due to the presence of 5 results situated below the LLOQ. Five months after a single injection of gadoterate meglumine, the Gd concentration decreased (0.004 nmol/g) and was
cies eluting after 20.1 minutes should be associated with molecules eluting before 13.1 minutes should be larger than 80 kDa, whereas species were eluted at 26.4 minutes. According to column calibration, all species were not included in the mass balance calculation.

After injection of gadoterate, at both M1 and M5, only one Gd species was observed to elute close to the LLOQ of the method (LLOQ = 0.002 nmol/g). At this time-point, the residual Gd concentrations in the cerebellum were 48-fold higher for gadobenate than for gadobenate.

**DISCUSSION**

This study was based on the methodological approach reported by Frenzel et al.\(^{13}\) to investigate whether residual Gd in the rat brain was present in the form of intact GBCA or in other chemical form(s) and Gd elimination from the brain parenchyma after repeated injections of different GBCAs (cumulative dose, 25 mmol/kg). A similar methodology was subsequently used in a 1-year study of the long-term brain elimination kinetics and Gd species in rats after 5 injections of GBCAs (cumulative dose, 12 mmol/kg).\(^{14}\) These analytical approaches are based on tissue fractionation and analysis of the water-soluble fraction extracted from different parts of the brain by HPLC-ICP-MS. In these studies, SEC was used for the detection of Gd species. Size-exclusion chromatography allows separation of analytes mainly according to their hydrodynamic volume, although other nonspecific mechanisms may also be concurrently involved. Smaller molecules flow more slowly through the column because they penetrate into the pores of the stationary phase, whereas large molecules are rapidly eluted through the column because they do not enter the pores. Hydrophilic interaction liquid chromatography, which allows unambiguous detection of small and hydrophilic molecules such as intact GBCA, was also used in 2 studies.\(^{14,15}\) Hydrophilic interaction liquid chromatography ICP-MS therefore provides complementary information to SEC-ICP-MS, allowing identification of intact GBCA in the soluble fraction but was not used in current study. These techniques allow a better understanding of Gd speciation and Gd elimination from the brain parenchyma after repeated injections of GBCAs. This study demonstrates, for the first time, the presence of different Gd species in rat cerebellum after a single injection of a clinically relevant dose of macrocyclic versus linear GBCAs. Because of very low brain Gd concentration expected after a single dose of 0.6 mmol/kg of GBCA, special precautions were taken to control for smaller than 12 kDa. The signal observed for gadoterate therefore corresponds to low molecular weight molecules, such as the form of GBCA initially injected. This retention time is in good agreement with the retention time detected for the standard GBCA solution.

| Contrast Agent | Type         | Extraction Efficiency (%) |
|----------------|--------------|---------------------------|
| Gadoterate meglumine | Macrocyclic | 70 ± 2 (n = 5) |
| Gadobenate dimeglumine | Linear | 25 ± 10 (n = 3) |
| Gadobenate | Linear | 8 ± 1 (n = 10) |

Differences in number of replicates are due to the fact that, for some samples, the Gd concentration in the supernatant was <LLOQ. Note that the LLOQ for the M5 point (0.002 nmol/g) were much lower than for M1 (0.018 nmol/g) because of a different ICP-MS system used.
the background level of Gd and to avoid cross-contamination at each step of the experimental work. The procedure used in this study proved to be robust for the analysis of a large number of complex biological samples containing trace levels of Gd. Regarding the outliers, one sample showed an extremely high Gd concentration, strongly suggesting a contamination during the experimental work for this sample. Some of the 5 other outliers may be due to the fact that, for the purpose of the statistical treatment, the LLOQ value was arbitrarily assigned to the concentrations below LLOQ, which may have induced ”artificial” outliers by the ROUT test.

Our data are qualitatively consistent with the results of previous studies of repeated injections in rats13–18 and with the study of single dose injection of GBCAs in a large animal model (ie, sheep).23 Linear GBCAs leave more residual total Gd in the cerebellum than macrocyclic GBCAs. Gadolinium elimination between M1 and M5 is much faster for the macrocyclic GBCA gadoterate (79% decrease in total, elementary Gd concentration) than for the linear GBCAs gadobenate and gadodiamide, with a 28% and 31% decrease, respectively, which confirms previous findings.14,16 Analysis of Gd speciation in the soluble fraction 1 month after a single injection of linear GBCAs showed that Gad was present in at least 2 distinct chemical forms: (1) small molecules including intact GBCA, and (2) soluble macromolecules. As reported by Robert et al.,14 we confirmed that the Gd detected in soluble macromolecules should be associated with the class of macromolecules larger than 80 kDa. However, we observed only one significant Gd species, whereas Robert et al reported 2 peaks. On the other hand, Frenzel et al suggested that only a single class of macromolecules contained Gd, but with significantly higher molecular weights (200–300 kDa). The different number of Gd signals bound to macromolecules, detected on SEC-ICP-MS chromatograms, could be explained by the use of different chromatographic columns. In this study, we used the same type of stationary phase as that used by Frenzel et al, which is a composite of cross-linked agarose and dextran, whereas Robert et al used a stationary phase composed of silica. The difference in molecular weight of the macromolecular Gd fraction detected (80 vs 300 kDa) is probably due to the use of different molecular weight markers for column calibration. It should also be noted that if the nature of the molecular weight marker used for column calibration differs from that of the analyte, molecular weights cannot be accurately calculated.

Compared with the Gd speciation observed for linear GBCAs, Gd was only detected in the form of intact GBCA after a single injection of the macrocyclic GBCA gadoterate. As previously described,14 washout of the intact GBCA can be observed for all GBCAs between M1 and M5. In the present study, we report an 89% to 100% decrease of the levels of the intact GBCA. In contrast, the amount of Gd bound to soluble macromolecules observed for gadobenate and gadodiamide remained constant 5 months after injection of a single dose of GBCA. It is possible that this fraction of Gd generates the persistent SI enhancement observed on MRI images. Consequently, the binding of Gd to soluble macromolecules could maximize its relaxivity and produce visible SI enhancement despite the very low Gd concentration. Another key point is that the extraction efficiency varies for each GBCA, as reported elsewhere.13,15,24 After injection of macrocyclic GBCAs, Gd was mostly found in the soluble brain fraction in the form of low molecular-weight molecules (Frenzel et al), corresponding to the intact GBCA form (Gianolio et al). Conversely, after injection of linear GBCAs, Gd was largely found in the pellet. The least thermodynamically stable GBCA could therefore induce other Gd forms, which are less effectively extracted by the sample preparation protocol, as reflected by our data. Interestingly, the extraction efficiency at M1 decreases in the order of stability of the GBCA: gadoterate > gadobenate > gadodiamide (Port et al).

A limitation of this study is the fact that SEC-ICP-MS does not allow identification of the chemical nature of the macromolecules and cannot demonstrate whether the intact linear GBCA or transmetalated Gd³⁺ ion is bound to macromolecules. This information would represent a substantial progress toward a better understanding of the processes involved in Gd accumulation in brain and the putative biological and toxicological consequences of this phenomenon. Frenzel et al suggested that intact GBCAs (except for gadobenate) do not bind to macromolecules because they do not bind to plasma proteins. Furthermore, a study comprising 15-day incubation of GBCAs in human serum showed release of 20% of free Gd³⁺ from nonionic linear GBCAs and 2% from ionic linear GBCAs, whereas no Gd release was reported for macrocyclic GBCAs.13 Although these findings strongly support the theory of potential dechelation of linear GBCAs, no evidence is available concerning the chemical form of Gd bound to macromolecules.

The second limitation of this study is that the speciation method only provides information about the water-soluble fraction. The insoluble fraction (pellet) that contains a large amount of Gd (especially for linear GBCAs) cannot be analyzed in terms of speciation. The pellet contains cell debris, inorganic precipitates such as colloidal forms of hydroxide, phosphate, and carbonate or denatured macromolecules, which could bind Gd or capture GBCAs. Research into solving this problem is already underway.

CONCLUSIONS

Even a single injection of a clinically relevant dose of Gd-based contrast agent resulted in long-term Gd detection in the cerebellar parenchyma of healthy rats. Administration of linear GBCAs (gadodiamide and gadobenate) resulted in higher residual Gd concentrations than
administration of the macrocyclic agent gadoterate (15-fold higher at M1 to 48-fold higher at M5 for gadodiamide vs gadoterate, 5-fold higher at M1 to 17-fold higher at M5 for gadobenate vs gadoterate). Speciation analysis of the water-soluble fraction of cerebellum confirmed washout of the intact GBCA form with time. The amount of Gd bound to macromolecules, only observed with the linear GBCA, remained constant 5 months after injection and likely represents a permanent deposition.

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