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Abstract

The aim of this work was to investigate, by five different administration protocols, the impact of the dosage, the time passed after the last injection and the frequency of injections, on accumulation and distribution of Gd-containing species in the body tissues of healthy mice upon repeated injections of Gadolinium Based Contrast Agents (GBCAs).

Gadodiamide and Gadoteridol have been compared. The amount of Gd retained in several tissues/organs (cerebrum, cerebellum, spleen, liver, kidneys, eyes, skin, bone and muscle) has been assessed by ICP-MS upon administration of the GBCAs i) at three weeks or three months after the last administration, ii) when one, three or twelve doses of GBCA were administered and iii) when administrations were made every two weeks.

Gd was found in all tissues after the administration of Gadodiamide. Conversely, in the case of Gadoteridol, Gd was detected only in spleen, kidneys, liver and bone. The amounts of Gd found in spleen, liver and kidneys markedly decrease upon increasing the time that has passed after the last administration, whereas, in the case of Gadodiamide, the decrease of Gd found in bone, cerebrum and cerebellum appears to occur at a much slower rate. Overall, areas of long term deposition appear to be bone and spleen for both GBCAs.

In conclusion, our findings demonstrate that intravenous multiple administrations of GBCAs is associated with extensive multiorgan retention which is reduced but not eliminated by the use of the macrocyclic Gadoteridol as well as by adopting reduced and/or less frequent dosing.

Keywords:

Gadolinium, Contrast Agents, Magnetic Resonance Imaging, metal retention

Abbreviations

ICP-MS – Inductively Coupled Plasma Mass Spectrometry

GBCAs – Gadolinium Based Contrast Agents

CNS – Central Nervous System

NSF - Nephrogenic Systemic Fibrosis

LOQ - Limit of quantification

FDA – Food and Drug Administration

Introduction

Gadolinium-based contrast agents (GBCAs) are routinely used in many clinical MRI protocols because of their ability to shorten the T_1 of water protons in the regions where they distribute. It has been estimated that about 40–50% of the current clinical MRI scans are contrast-enhanced[1]. The use of GBCAs is particularly important in studies involving the Central Nervous System (CNS) (e.g. multiple sclerosis, cerebral injuries, neurological cancers etc.) [2-4]. To avoid the release of gadolinium ions, multidentate ligands have been selected for the design of MRI contrast agents. The currently used GBCAs are made by linear or macrocyclic polyaminocarboxylic acid ligands that tightly chelate the paramagnetic Gd^{3+} ion. The stability constants of macrocyclic Gd-based complexes are in the order of 10^{21}-10^{25} whereas for linear complexes they are in the order of 10^{16}-10^{23} [5]. In a typical diagnostic protocol, GBCAs are administered intravenously at the dose of 0.1 – 0.3 mmol Gd^{3+}/kg body weight. They distribute rapidly in the vascular and extracellular space and their excretion occurs, for the systems that do not bind to plasma proteins, through the kidneys via glomerular filtration. The half-life of elimination is generally 1.3–1.6 hours in patients with normal kidney function [6]. The high stability should ensure that the Gd-complexes are excreted intact without side effects for patients. However, it was shown that, when a severe renal impairment is present, the elimination half-life may raise up to 30-40hours. As a consequence of this long circulation time, a partial dissociation of the metal chelates may occur with the release of Gd^{3+} ions that may be taken-up by biomolecules. In the past decade, it has been reported that the release of Gd^{3+} ions from

GBCAs can trigger the onset of a severe systemic disease, called Nephrogenic Systemic Fibrosis (NSF), which is a rare and serious syndrome that involves fibrosis of skin, joints, eyes, and internal organs [7-9].

More recently, it has been reported that tiny amounts of Gd^{3+} are retained in tissues also in absence of renal dysfunction [10,11]. Successive studies confirmed the early reports showing that Gadolinium accumulates in the brain, particularly in the dentate nucleus and globus pallidus, where an increased signal intensity on unenhanced T1-weighted MRIs was observed [12]. Besides the few observations in humans, a number of studies on animal models have been carried out [13-19]. Recently, we reported that, in the case of multiple administrations of Gd-DTPA-BMA, only about 18% of the total Gd retained in the cerebellum is in the form of intact complex [14].

Starting from the above reported considerations, the aim of the herein reported study was to investigate the in vivo fate of two clinically approved GBCAs, namely Gadoteridol (ProHance, Bracco) and Gadodiamide (Omniscan, GE Healthcare). In particular, the aim of the study was to extend the investigation of Gd retention to other body tissues besides brain in order to get a comprehensive view which could help in the understanding of Gd accumulation/excretion pathways. Moreover, the study has dealt with the comparison of administration protocols differing for i) the number of total doses, ii) the frequency of the administrations and iii) the sacrifice time after the last administration.

Materials and methods

Chemicals

Two Gd-based contrast agents (GBCAs) were used for this study, namely Gadodiamide (Gd-DTPA-BMA, Omniscan 0.5 mol/L; GE Healthcare, Little Chalfont, UK) and Gadoteridol (Gd-HPDO3A, ProHance 0.5 mol/L; Bracco Imaging, Milan, Italy).

Animals

Eight-week-old male Balb/c mice (Charles River Laboratories, Calco, Italy) were used for the *in vivo* experiments (n=5 for each study, mean weight 24±1 g). Mice were kept in standard housing with standard rodent chow and water available *ad libitum*, and a 12h light/dark cycle. Experiments were performed according to the national laws on experimental animal (L.D. 26/2014; Directive 2010/63/EU) and were approved by the Italian Health Ministry (Direzione Generale della sanità animale e dei farmaci veterinati, prot. n° 808/2017-PR). Before the injection of the GBCAs, mice were anesthetized by intramuscular injection of tiletamine/zolazepam (Zoletil 100; Virbac, Milan, Italy) 20 mg/kg plus xylazine (Rompun; Bayer, Milan, Italy) 5 mg/kg.

GBCAs administration protocols

Each GBCA (Gadodiamide or Gadoteridol) was intravenously administered at the dose of 0.1mmol/kg *via* the tail vein (following the FDA guidance [20], this dose corresponds to a dose of 0.008mmol/kg in humans). Five injection protocols have been used, as reported in Fig.1 *i.e.* : A) 12 *i.v.* administrations 3-times for week, animal's sacrifice 21 days after the last GBCA dose; B) 3 *i.v.* administrations in a week, animal's sacrifice 21 days after the last GBCA dose; C) 1 administration, animal's sacrifice 21 days after the single GBCA dose; D) 12 administrations 3-times for week, animal's sacrifice 3 months after the last GBCA dose; E) 12 administrations every two weeks, animal's sacrifice 21 days after the last GBCA dose

ICP-MS Measurements of metal content

At the end of each experiment, the animals were sacrificed and the following organs/tissues were explanted: cerebrum, cerebellum, liver, spleen, kidneys, eyes, muscle, bone and skin. The explanted specimens were weighed, lyophilized and processed for ICP-MS. For this purpose, 1 ml of concentrated HNO_3 (70%) was added to each lyophilized sample. After complete dissolution of the tissues, samples were further digested by applying microwave heating (MicroSYNTH, Microwave labstation equipped with an optical fiber temperature control and HPR-1000/6M six position high-pressure reactor, Milestone, Bergamo, Italy). After digestion, the volume of each sample was brought to 2 ml with ultrapure water, filtered with 0.45 μm filter and analyzed by ICP-MS, using a Thermo Scientific ELEMENT 2 ICP-MS -Finnigan, Rodano (MI). The quantification was obtained through a calibration curve measured by using four gadolinium absorption standard solutions (Sigma-Aldrich) in the range 0.005–0.1 μg/ml.

The total mass of Gd^{3+} retained in each specimen was calculated in respect to the weight of dry tissue (as μg of Gd^{3+}/g of dry tissue).

The limit of quantification (LOQ) in our ICP-MS analyses was determined to be 0.0005 μg/mL (100 times higher than blank solution). Considering the mean weights of the organs/tissues recovered from mice and the dilutions made in the preparation of the samples for ICP/MS, the LOQ of the method with respect to the respective tissues/organs were: liver 0.0049 μg/g, spleen 0.0300 μg/g, kidney 0.0088 μg/g, muscle 0.0192 μg/g, cerebrum 0.0171 μg/g, cerebellum 0.0370 μg/g, bone 0.0156 μg/g, skin 0.0694 μg/g.

Statistical Analysis

All data are expressed as mean ± standard deviation. Between-group differences with respect to the mean Gd concentration of GBCA were assessed using unpaired t-test. The Graph-Pad Prism software was used for data analysis. A P value < 0.05 was considered to be statistically significant.

Results

The herein reported results deal with the retention of Gd in tissues and organs after multiple administrations of GBCAs in healthy BALB/C mice. Even if the FDA (Food and Drug Administration) guidance with respect to the adaptation of the administered dose on the basis of the body surface/weight would suggest to use a higher dose for mice compared to that used in humans [18], we decided to explore the issue of Gd retention even at the lower limit of the administered dose, thus each injection was made with 0.1 mmol Gd /Kg and different administration protocols were compared.

Two GBCAs have been compared, namely Gadodiamide (Omniscan, Gd-DTPA-BMA) and Gadoteridol (ProHance, Gd-HPDO3A), as representative of linear neutral and macrocyclic neutral chelates categories, respectively.

Long-time Gd-retention in tissues/organs upon repeated administrations of GBCAs

The applied experimental work-up (protocol A, Fig.1) consists of a total Gd dose of 1.2 mmol/Kg, administered in 12 i.*v*. injections (0.1 mmol/Kg for each dose). 21 days after the last administration, the total Gd concentration (in μg/g of dry tissue) has been determined by ICP-MS analysis. Results are reported in Fig.2 and table 1.

The amounts of Gd found in the examined tissues (cerebrum, cerebellum, eye, muscle, bone, spleen, liver, kidneys and skin) after Gadodiamide administrations are significantly higher than in control mice (where they were all under the limit of quantification (LOQ)). Conversely, in the case of Gadoteridol administrations, the difference is significant only in spleen, liver, kidney and bone.

Overall, the amounts of Gd retained in these four organs result to be ca. one order of magnitude higher in mice treated with Gadodiamide than in those treated with Gadoteridol.

An interesting finding deals with the markedly high Gd levels in spleen (6.25±0.733 μg/g tissue for Gadodiamide and 0.616±0.122 μg/g tissue for Gadoteridol). These values are three times higher than the amount found in bone and ca. 20 times larger than that measured in liver in the case of Gadodiamide and ca. ten times higher than those found in bones and liver in the case of Gadoteridol. Conversely, the amounts of retained Gd in cerebrum, cerebellum and eye are similar to those found in muscle and skin tissues.

Effect of the number of administrations on tissue/ organ Gd-accumulation

Next, the effect of varying the number of administrations (namely 12, 3 or 1), i.e. the total dose (1.2, 0.3 or 0.1 mmol/Kg, respectively), on the amount of Gd retained in the different tissues/organs has been assessed. Results are reported in Fig.3. The amount of retained Gd appears directly dependent on the number of doses for both GBCAs. The individual values ± SD and the statistical differences in each tissue/organ are detailed in Fig S1 and Fig S2 for Gadodiamide and Gadoteridol, respectively. As reported in the graphs, in the case of Gadodiamide administration, 3 doses are sufficient to obtain, after 21 days from the last administration, a significant retention in all the investigated tissues/organs but cerebellum (Fig.3 and Fig.S1). Upon Gadoteridol administration, the Gd-distribution in the different tissues/organs follows what observed for Gadodiamide although the detected Gd quantities are one order of magnitude lower (Fig.3 and Fig.S2). Finally, a single administration appears sufficient to yield a detectable Gd retention in all the organs devoted to body clearance (liver, spleen and kidneys) and in bones, for both Gadodiamide and Gadoteridol, in respect to controls, when animals are sacrificed 21 days after the administration of the GBCAs.

Elimination of Gd from tissues/organs

Figure 4A compares the results obtained from the application of protocols A and D in which the same total dose of GBCAs is administered (1.2 mmol/kg in 12 doses 0.1 mmol/kg each, three times weekly). The two protocols differ for the time elapsed between the last administration and the animals' sacrifice (i.e. 3 weeks or 3 months for the protocol A and D, respectively). In general, the amount of retained Gd is lower when measured at longer time from the administration, for both Gadodiamide and Gadoteridol. Anyway, even three months after the administration of the last dose, the amount of detected Gd continues to be significantly higher than in control mice in all the tissues/organs, in the case of Gadodiamide, and in bones and spleen, in the case of Gadoteridol.

Effect of the frequency of administrations in Gd-retention

Finally, the frequency of the administrations of GBCAs has been evaluated. The same total dose (1.2 mmol/Kg) of Gadodiamide or Gadoteridol has been given to mice via i.v. administrations that occurred every two days (protocol A) or every two weeks (protocol E). The results are reported in Figure 5. An overall decrease in the amount of retained Gd is observed, for all the investigated tissues/organs, both in the case of mice administered with Gadodiamide and Gadoteridol, when the doses are diluted over a longer period.

From Fig.S4, it is evident that, in the organs where the "high frequency" protocol (A) triggers the accumulation of significant amounts of Gd, a lower, but still significant, accumulation is also present upon applying of the "low frequency" protocol (E).

Discussion

Several animal studies have been reported in the last two years aimed at gaining a better understanding of the determinants of the accumulation of Gadolinium in the brain upon GBCAs administration. Herein, the investigation has been extended to other body tissues and organs. Moreover, the study included the comparison of several administration protocols differing in the number of total injections, doses, frequency of administrations and the sacrifice time after the last administration.

In general, the amounts of retained Gd resulted to be one order of magnitude higher for animals treated with Gadodiamide in respect to the animals treated with Gadoteridol. Furthermore, using Gadodiamide, Gd resulted to accumulate in all the investigated tissues/organs, whereas, in the case of Gadoteridol, Gd is found only in the spleen, liver, kidney and bone (Fig.2 and table 1, 12 administrations and sacrifice 21 days after the last dose). This result appears in line with previous studies in which the two complexes were compared [14,15]. In analogy to what suggested for a GBCA that has crossed the BBB, here too one may expect that the fate of the administered agent is determined by a number of factors such as the composition of the extravascular medium, the thermodynamic and kinetic stability of the GBCA, etc. [5,21].

When the effect of received dose (12, 3 or 1 injections to give a total dose of 1.2, 0.3 and 0.1 mmol/Kg) on the amount of Gd retained in the different tissues/organs was investigated, it was found that the amount of Gd decreases by lowering the dose, for both the GBCAs. However, even one administration appears sufficient to measure a detectable amount of Gd in the organs devoted to body clearance (liver, spleen and kidney) and in bones for both the linear and macrocyclic complexes. Over time, these three organs tend to eliminate the retained Gd, but the accumulation of residual gadolinium in bones reveals that gadolinium deposited in this region can persist for long time.

The capability of removing Gd from tissues/organs after dosing has been tested by comparing results obtained after 3 weeks or 3 months from the last administration. Overall, a decrease in the amount of retained Gd is observed for both Gadodiamide and Gadoteridol, but, even 3 months after the last dose, detectable amounts of Gd persist in all the tissues/organs, in the case of Gadodiamide, and in bones and spleen, in the case of Gadoteridol. This result underlines the occurrence of slow excretion kinetics for gadolinium retained in the body, particularly in the case of Gadodiamide. The retention of Gd in human bones upon administration of GBCAs (Gadodiamide and Gadoteridol) has been early pointed out in papers published before that the issue of Gd retention in the brain was evidenced. Gibby et al [20,21] demonstrated that Gadodiamide left 4 times more Gd in bone than Gadoteridol, and

Darrah et al [22] confirmed that, in patients with documented exposure to Gadodiamide and Gadoteridol, Gd is deposited into bones and is retained for more than 8 years. Our results confirm that bone tissues of animals exposed to GBCAs have Gd concentrations that are significantly higher than in control non-exposed mice, indicating that Gd is incorporated into bone mineral and does not completely clear the body even at very long times. Noticeably, even if to a less extent, bone Gd retention is observed also upon injection of the highly stable Gadoteridol. This finding calls for the need of further research to determine the chemical form of Gd incorporated into bone.

The concentration of Gd retained in the brain regions, in the case of mice administered with Gadodiamide, 3 months after the last dose is still significantly higher than in control mice and is in agreement with the values reported from Smith and coworkers [25] for rats 5 months after dosing. In their work, a histopathologic analysis of the brain specimens was carried out to conclude that no-histopathologic abnormalities were observed.

One of the most important results from the herein reported study deals with the remarkably high Gd levels in spleen. This finding, together with the observation of long retention time, may provide new insights on how Gd distributes over time in the body tissues.

One may speculate that a potential reservoir for a prolonged delivery of Gd containing systems may be represented by spleen. This organ is committed to the processing of xenobiotics (as GBCAs) operating like a “large” lymph node. The observed behavior appears to parallel what recently reported in a study devoted to the comparison between La-DTPA and Gd-DTPA where spleen is the organ more involved in the accumulation of both La- and Gd-containing species [26].

One of the criticisms which is frequently raised in the case of studies on Gd-retention carried out on animal models is that, in order to induce Gd accumulation in relatively short experimental times, very high doses of GBCAs are administered one after the other. In order to meet conditions closer to those

applied in the clinics on humans, we set up procedures where the administered doses were diluted over a longer period of time.

We compared the results obtained when the same total dose of Gadodiamide or Gadoteridol was administered to mice every two days (protocol A) or every two weeks (protocol E).

Generally, a decrease in the amount of retained Gd is observed for all the investigated organs when the doses are diluted over a longer period of time. This difference is particularly evident in the spleen. Anyway, in all the investigated tissues/organs, even if the total Gd concentration is lower when protocol E is used, still a significant difference is observed with respect to control mice in all the cases in which it was observed by using protocol A. Thus, it can be concluded that the protocols generally used in this kind of animal studies, which rely on the use of frequent administrations, can be considered qualitatively reliable even if they can lead to an overestimation of Gd-retention.

The two used GBCAs display chemical analogies as both are small molecules, non-ionic and hydrophilic. Our and others' observations point towards an analogous ability to enter the brain [26,27] and accumulate in the extravascular/ extracellular compartment in the brain as well in all the other tissues/organs considered in this work. Now, the larger amounts of retained Gd in the case of Gadodiamide likely suggest that the "journey" of the GBCA is affected by its lower kinetic and thermodynamic stability. Said that, no much can be added as no relevant histological/pathological change has yet been related to the occurrence of retained Gd.

In conclusion, our findings demonstrate that intravenous multiple administrations of GBCAs is associated with extensive multiorgan retention which is reduced but not eliminated by the use of the macrocyclic Gadoteridol as well as by adopting reduced and/or less frequent dosing.

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Compliance with Ethical Standards: Experiments were performed according to the national laws on experimental animal (L.D. 26/2014; Directive 2010/63/EU) and were approved by the Italian Health Ministry (Direzione Generale della sanità animale e dei farmaci veterinati, prot. n° 808/2017-PR).

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Table 1: Gd concentrations (in μg/g of dry tissue) for the various tissues/organs determined by ICP-MS analysis upon application of the experimental protocol A. (Mean values ± SD). LOQ= Limit of Quantification

Tissue/organ	Control group (µg/g of dry tissue)	Gadodiamide group (µg/g of dry tissue)	Gadoteridol group (µg/g of dry tissue)
Cerebrum (LOQ=0.017)	< LOQ	0.231±0.026	< LOQ
Cerebellum (LOQ=0.037)	< LOQ	0.302±0.034	< LOQ
Liver (LOQ=0.005)	< LOQ	0.315±0.051	0.079±0.018
Spleen (LOQ=0.030)	< LOQ	6.250±0.733	0.616±0.122
Kidney (LOQ=0.009)	< LOQ	1.830±0.318	0.083±0.017
Muscle (LOQ=0.019)	< LOQ	0.181±0.018	< LOQ
Bone (LOQ=0.015)	< LOQ	2.170±0.183	0.063±0.009
Eye (LOQ=0.092)	< LOQ	0.286±0.138	< LOQ
Skin (LOQ=0.069)	< LOQ	0.102±0.060	< LOQ

Figure Legends:

Figure 1: Scheme of the experimental protocols; Each study has dealt with 2 groups (n=5 each) of mice administered with 0.1 mmol/Kg of Gadoteridol or Gadodiamide according to the following protocols: Protocol A, 12 doses administered every two days and sacrifice 21 days after the last dose; Protocol B, 3 doses administered every two days and sacrifice 21 days after the last dose; Protocol C, 1 dose and sacrifice 21 days after the last dose; Protocol D, 12 doses administered every two days

and sacrifice 3 months after the last dose; Protocol E, 12 doses administered every two weeks and sacrifice 21 days after the last dose.

Figure 2: Results from the application of protocol A. Total Gd concentrations are reported in μg per gram of dry tissue. Individual values, mean, SD and statistical difference with respect to tissues/organs from untreated mice are given.

Figure 3: Effect of the number of administered doses on the amount of Gd retained in the different tissues/organs for Gadodiamide and Gadoteridol, respectively (comparison of protocols A, B and C). Data are reported as mean±SD.

Figure 4: Effect of the time elapsed after the last administered dose on the amount of Gd retained in the different tissues/organs for Gadodiamide and Gadoteridol, respectively (comparison of protocols A and D). Data are reported as mean±SD.

Figure 5: Amounts of Gd retained in the different tissues/organs after the administration of 12 doses (0.1 mmoles/Kg) of Gadodiamide or Gadoteridol every two days (protocol A) or every two weeks (protocol E), respectively. Data are reported as mean±SD.

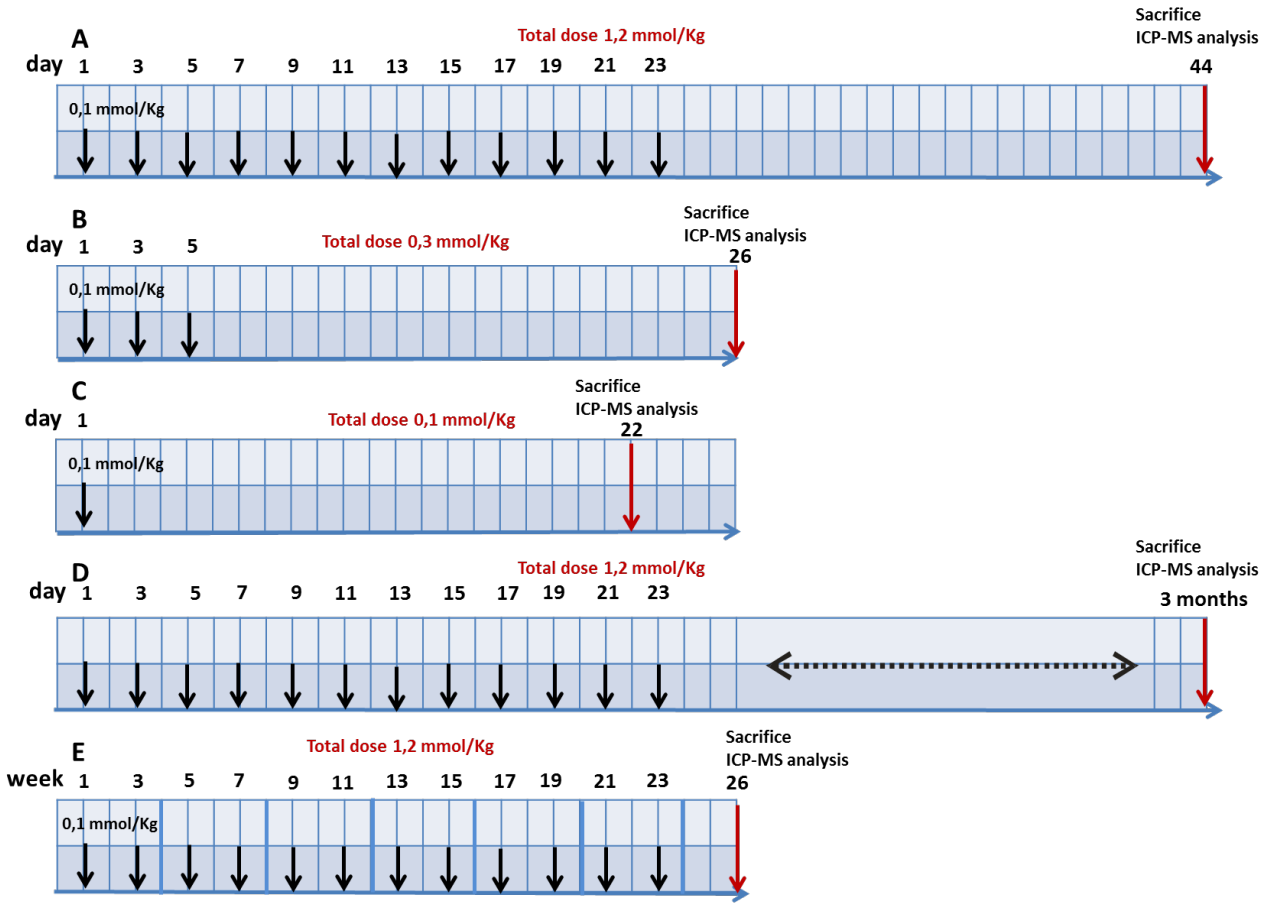
A
day 1 3 5 7 9 11 13 15 17 19 21 23
Total dose 1,2 mmol/Kg
Sacrifice
ICP-MS analysis
44
0,1 mmol/Kg
B
day 1 3 5
Total dose 0,3 mmol/Kg
Sacrifice
ICP-MS analysis
26
0,1 mmol/Kg
C
day 1
Total dose 0,1 mmol/Kg
Sacrifice
ICP-MS analysis
22
0,1 mmol/Kg
D
day 1 3 5 7 9 11 13 15 17 19 21 23
Total dose 1,2 mmol/Kg
Sacrifice
ICP-MS analysis
3 months
E
week 1 3 5 7 9 11 13 15 17 19 21 23
Total dose 1,2 mmol/Kg
Sacrifice
ICP-MS analysis
26
0,1 mmol/Kg

Figure 1

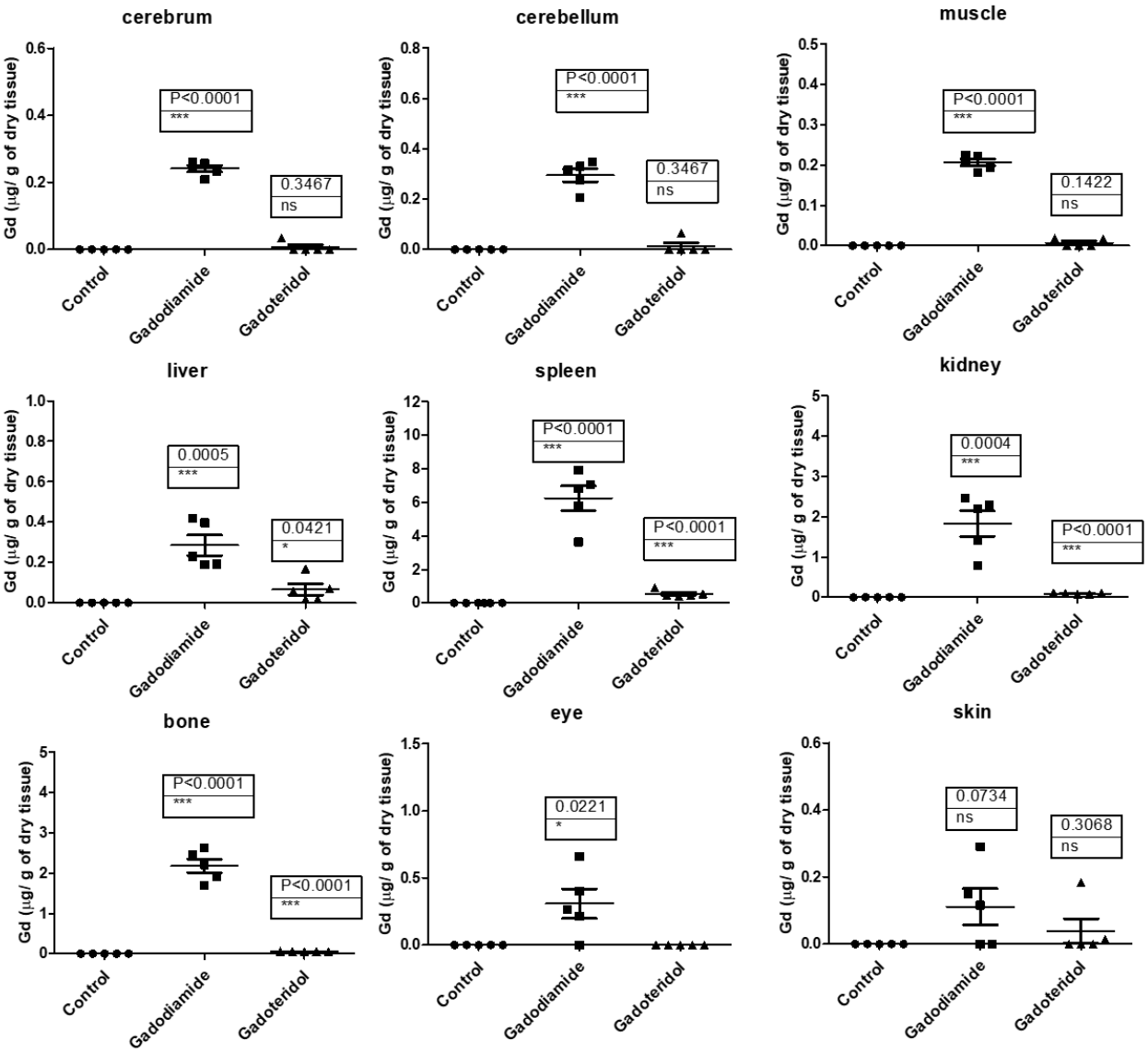
cerebrum
Gd (µg/ g of dry tissue)
P<0.0001
***
0.3467
ns
Control
Gadodiamide
Gadoteridol
cerebellum
P<0.0001
***
0.3467
ns
muscle
P<0.0001
***
0.1422
ns
liver
0.0005
***
0.0421
*
spleen
P<0.0001
***
P<0.0001
***
kidney
0.0004
***
P<0.0001
***
bone
P<0.0001
***
P<0.0001
***
eye
0.0221
*
skin
0.0734
ns
0.3068
ns

Figure 2

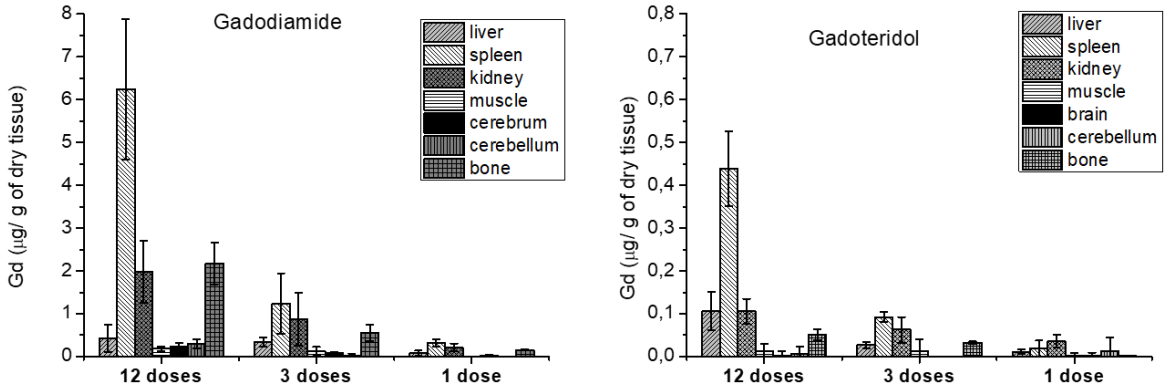
Gadodiamide
Gadoteridol
Gd (µg/ g of dry tissue)
liver
spleen
kidney
muscle
brain
cerebellum
bone
12 doses
3 doses
1 dose

Figure 3

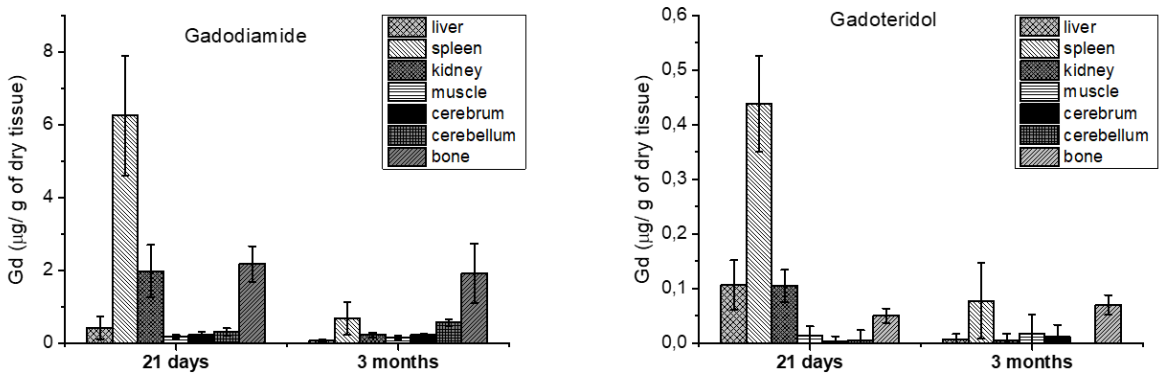

Figure 4

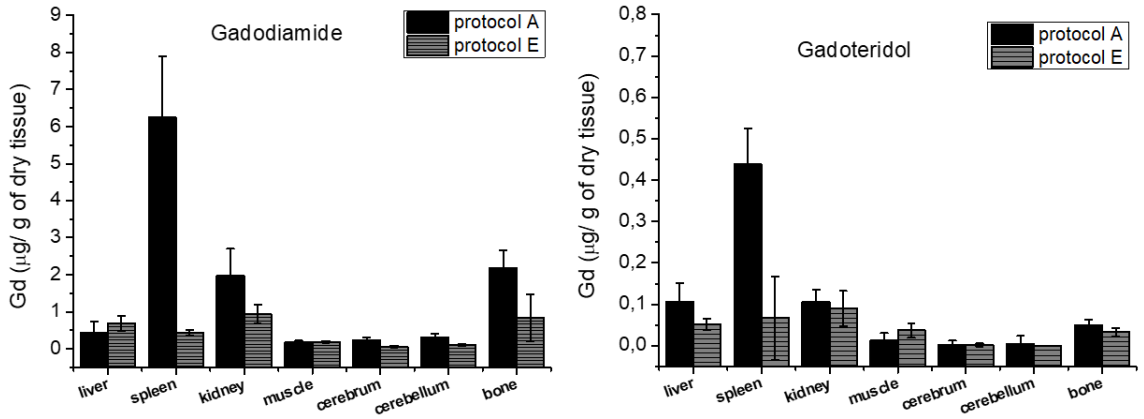

Figure 5