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ANALYSIS

DETERMINATION OF STABILITY CONSTANTS AND ACUTE TOXICITY OF POTENTIAL HEPATOTROPIC GADOLINIUM COMPLEXES

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Abstract: Due to their high specificity for the hepatobiliary system, iminodiacetic acid derivatives are known to form a class of hepatobiliary agents. In this paper we present new hepatotropic gadolinium complexes to be used as potential MRI contrast agents. Derivatives of N-(2-phenylamine-2-oxoethyl)iminodiacetic acid are introduced as ligands into such complexes. In this way, we hope to achieve a valuable diagnostic tool for investigating of pathological changes in the liver. Stability constants of complexes were determined by potentiometric titration in 0.1 mol L^{-1} $NaNO_3$ solution at 20.0 ± 0.1°C. Stability and selectivity constants were also determined for endogenous metal ions such as Cu^{2+}, Ca^{2+}, and Zn^{2+} with the use of SUPERQUAD computer program. Acute toxicity of new gadolinium complexes was assessed in mice and histopathology examinations were carried out.

Keywords: gadolinium complexes, hepatotropic contrast agents, stability constants, toxicity, histopathology

Magnetic resonance imaging (MRI) has become an important tool in routine clinical liver imaging. The complex $[Gd(DTPA)(H_2O)^{2-}]$ has been approved as the first clinically used contrast agent decreasing proton relaxation time in MRI (1, 2). Since that time, lanthanides complexes have been the subject of extensive research. Nowadays, complexes of gadolinium ion play the most important role in clinical use as contrast agents in MRI. Their paramagnetic properties result from the presence of gadolinium, having seven unpaired electrons and revealing strong relaxation effects (3, 4).

However, in the quantities required for clinical use, free gadolinium ions Gd^{3+} are highly toxic. In order to reduce its toxicity, gadolinium ion must be transformed into complex with an organic molecule (5-7). Evaluation of any new gadolinium complexes as potential MRI contrast agent should be focused on stability and kinetic behavior. One has to remember that complexes contain relatively toxic components: metal ion and ligand, bound by ionic forces (8). Moreover, under *in vivo* conditions, gadolinium ion present in the complex may be replaced with protons or endogenous metal ions such as Cu^{2+}, Zn^{2+} or Ca^{2+}, during spontaneous dissociation, or transmetalation *via* competitive reactions (9).

One of the most important properties of contrast agents is the safety of their usage, but contrast agents should also display highly effective impact on selected organs. Gadolinium-based agents, such as Gd-DTPA (gadopentetate dimeglumine, Magnevist®, Schering AG), previously approved for clinical use, are not sufficiently specific and selective. Other Gd^{3+} based contrast agents, similar to Magnevist: Gd-BOBTA (gadobenate dimeglumine, Multihance®, Bracco Imaging) and Gd-EOB-DTPA (gadoxetate disodium, Primovist®, Schering AG), are more lipophilic and are selectively taken up by hepatocytes (10-12). Currently, non-invasive stimulation of the morphology and functions of the biliary ducts is not possible. In NMR diagnosis of liver and biliary ducts diseases, hepatotropic contrast agents enabling identification and differentiation of focal changes in liver and determination of reasons for cholestasia are still in demand.

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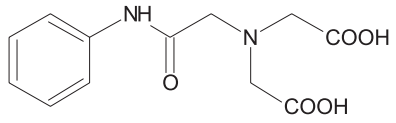

Figure 1. Structure of 2,2'-(2-phenylamino-2-oxoethyl)iminodiacetic acid

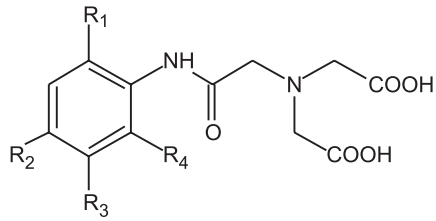

Figure 2. Structure of ligands

	R_1	R_2	R_3	R_4
L1	CH_3	CH_3	H	CH_3
L2	CH_3	H	H	CH_3
L3	CH_3	CH_3	Br	CH_3
L4	H	H	H	H

In the paper we present potential hepatotropic gadolinium complexes, with no pharmacologic data attached. The ligands are derivatives of N-(2-phenylamine-2-oxoethyl)iminodiacetic acid (Figures. 1, 2). Due to their high specificity for hepatobiliary system, derivatives of iminodiacetic acid are known to form a class of hepatobiliary agents (13).

The objective of the study was to determine stability constants of complexes formed by ligands (L1, L2, L3, L4) and metal ions Gd^{3+}, Cu^{2+}, Ca^{2+}, Zn^{2+}, as well as acute single dose toxicity of new potential hepatotropic gadolinium complexes.

EXPERIMENTAL

Chemicals

Synthesis of chloroacetanilide (**2a-2c**)

Aniline solution (**1a**) (24 mL, 0.27mol) in acetone (200 mL) was placed in an ice bath and stirred for 10 min. Chloroacetyl chloride (16 mL, 0.13 mol) was added dropwise into the solution, and it was stirred for 5 h at room temperature. Then, the precipitate was isolated by crystallization from water. Two recrystallizations and drying in dessicator over P_2O_5 yielded 93.39% of **2a**: m.p. 125-128°C; ^{1}H NMR (300 MHz, DMSO-d_6) (δ, ppm): 10.29 (1H, s, -NHCO-), 7.57, (2H, dd, *o*-H-Ph), 7.33 (2H, t, *m*-H-Ph), 7.09 (1H, t, *p*-H-Ph), 4.25 (2H, s, -CH_2-). Analysis for C_8H_8NOCl (calc.) found : %C (56.65) 56.69; %H (4.75) 4.55; %N (8.26) 8.24. Repeating the same procedure with **1b and 1c** gave **2b and 2c**, respectively. **2b**: yield: 94.37%; m.p. 150-153°C; **2c**: yield: 90.83%; m.p. 178-180°C; ^{1}H NMR (300 MHz, DMSO-d_6) (δ, ppm): 9.53 (1H, s, -NHCO-), 6.78 (2H, s, *m*-H-Ph), 4.25 (2H, s, -CH_2-), 2.22 (3H, s, *p*-CH_3Ar), 2.08 (6H, s, *m*-CH_3Ar). Analysis for $C_{11}H_{14}NOCl$ (calc.) found: %C (62.12) 62.46; %H (7.10) 7.03; %N (6.59) 6.58.

Synthesis of N-(2-phenylamine-2-oxoethyl)iminodiacetic acid (**3a-3c**)

A solution of chloroacetanilide (10 g, 0.05 mol, **2a**) and iminodiacetic acid sodium salt (6.7 g, 0.05 mol) in ethanol-water mixture (3:2 v/v) was refluxed for 5 h at 90-95°C, with pH being adjusted every hour to 12 with 10% NaOH solution. The mixture was cooled to room temperature and then pH was adjusted to 3.0 by the addition of conc. HCl. The precipitate was isolated by filtration. Its recrystallization from ethanol-water mixture (1:1 v/v, 3 times) gave **3a** with 65% yield and m.p. 158-160°C; ^{1}H NMR (300 MHz, DMSO-d_6) (δ, ppm): 12.60 (2H, s, -COOH), 10.26 (1H, s, -NHCO-), 7.61 (2H, dd, *o*-H-Ph), 7.32 (2H, t, *m*-H-Ph), 7.08 (1H, t, *p*-H-Ph), 3.56 (4H, s, -CH_2-), 3.48 (2H, s, -CH_2-). Analysis for $C_{12}H_{14}N_2O_5$ (calc.) found: %C (54.13) 55.09; %H (5.30) 5.29; %N (10.52) 10.24.

Repeating the same procedure with **2b** and **2c** gave **3b** and **3c,** respectively. **3b**: yield: 92.54%; m.p. 178-183°C; ^{1}H NMR (300 MHz, DMSO-d_6) (δ, ppm): 12.51 (2H, s, -COOH), 6.87-6.90 (3H, m, ArH), 4.16 (s, 2H, $NHCOCH_2N$), 3.65 (4H, s, 2 NCH_2CO_2), 2.25 (s, 6H, 2 *m*-CH_3Ar). Analysis for $C_{14}H_{18}N_2O_5$ (calc.) found: %C (57.14) 57.75; %H (6.16) 6.21; %N (9.52) 9.89. **3c**: yield: 90.77%; m.p.: 182-184°C; ^{1}H NMR (300 MHz, DMSO-d_6) (δ, ppm): 12.59 (2H, s, -COOH), 9.53 (1H, s, -NHCO-), 6.88 (2H, s, 2 *m*-H-Ph), 3.57 (4H, s, 2 NCH_2CO_2), 3.48 (2H, s, -CH_2-), 2.22 (3H, s, *p*-CH_3Ar), 2.09 (6H, s, *o*-CH_3Ar). Analysis for $C_{15}H_{20}N_2O_5$ (calc.) found: %C (58.14) 58.65; %H (6.54) 6.91; %N (9.08) 8.89.

N-(3-bromo-2,4,6-trimethylacetanilide)iminodiacetic acid (**3d**) was synthesized according to the patented method (21): Yield: 78%; m.p. 197-198°C; ^{1}H NMR (300 MHz, DMSO-d_6) (δ, ppm): 12.61 (2H, s, -COOH), 7.11 (5H, s, -Ph), 3.57. Analysis for $C_{15}H_{19}N_2O_5Br$ (calc.) found: %C (46.48) 46.74; %H (4.91) 4.89; %N (7.23) 7.13.

General procedure for the synthesis of gadolinium complexes with N-(2-phenylamine-2-oxoethyl)iminodiacetic acid sodium salt (**4b-4d**)

N-(2-phenylamine-2-oxoethyl)iminodiacetic acid (3.64 g, 0.014 mol) was dissolved in 10 mL of methanol, and added to LiOH solution (0.67 g, 0.028 mol). The mixture was stirred for 24 h at room temperature. Then, $GdCl_3$ solution (2.17 g; 0.006 mol) was added. The resulting solution was stirred for 24 h at 60°C, followed by dropwise addition of 1 mL of 10% NaOH solution. After 30 min methanol was evaporated. Recrystallization from methanol/acetonitrille mixture (1:4, v/v) gave **4a** with 96.28% yield. Analysis for $C_{24}H_{24}N_4O_{10}GdNa$ (calc.) found: %C (33.22) 33.61; %H (4.29) 4.02; %N (5.99) 5.97. Repeating the same procedure with **3b-3d** yielded **4b-4d**, respectively. **4b**: yield 94.7%; m.w.: 764.8. Analysis for $C_{28}H_{32}N_4O_{10}GdNa$ (calc.) found: %C (36.32) 36.07; %H (5.87) 5.67; %N (6.04) 6.07. **4c**: yield 93.6%. Analysis for $C_{30}H_{36}N_4O_{10}GdNa$ (calc.) found: %C (37.08) 37.21; %H (4.90) 4.48; %N (5.24) 5.22. **4d**: yield 98.6%. Analysis for $C_{30}H_{36}N_4O_{10}BrGdNa$ (calc.) found: %C (33.85) 33.22; %H (3.94) 4.19; %N (5.98) 5.96.

Ligands solutions were prepared by dissolving weighed portions of appropriate ligands in purified water (Millipore, Elix3). The final concentrations of ligands solutions were ca. 0.006 mol L^{-1}. Metals ion solutions (ca. 0.003 mol L^{-1}) have been prepared from $GdCl_3 \times 6\ H_2O$ (99.9%, Aldrich), $CaNO_3$, $CuCl_2$, $ZnCl_2$ (POCh, Gliwice, Poland), and their concentration values were confirmed by colorimetric titration with the use of standard EDTA solution (POCh, Gliwice, Poland). The 0.1 mol L^{-1} standard solution of carbonate-free sodium hydroxide (DILUT-IT® J.T. Baker, Deventer Holland) was prepared.

Potentiometric titrations

Equilibrium constants of both protonation and metal complexation reactions were determined with pH-potentiometric titration. To this end, the combined glass electrode BlueLine (Schott, Germany), the pH-Meter CG 842 (Schott-Geräte, Germany) and the Solarus Digital Titrator (Hirschmann Laborgeräte, Germany) were used. The electrode was used after previous calibration with two buffers of pH = 4.00 and 7.00 (± 0.05), by titrating 0.01 mol L^{-1} HCl solution with NaOH at the ionic strength of I = 0.1 ($NaNO_3$), under nitrogen atmosphere.

All experiments were carried out in a titration cell thermostated at 20.0 ±0.1°C in aqueous solution under nitrogen as inert gas, to exclude carbon dioxide. The ionic strength was adjusted with 0.1 mol L^{-1} $NaNO_3$ solution (J.T. Baker, Deventer Holland) and 0.1 mol L^{-1} sodium hydroxide solution was used as titrant. Prior to the titration, small amounts of HCl were added in order to adjust the solution to pH 2.5. The titrations were carried out at 2:1 of ligand to metal ion concentration ratio. Protonation of ligands and stability constants of complexes were calculated using SUPERQUAD program.

Acute toxicity and histopathological studies

Animal handing routies were performed according to Good Laboratory Practice. Research protocol of animal experiments was approved by the "Institutional Animal Ethics Committee" of Medical University of Łódź (permit No. Ł/BD/231). Acute toxicity studies on gadolinium complexes, which are the subject of this paper, were performed on Swiss albino mice of either sex. Each complex was administered in a single dose of 2000 mg/kg, either by gavage, with an oral dosing needle or intraperitoneally in a dose of 200 mg/kg. Each particular dose-obtaining group contained 8 animals (4 males and 4 females). The animals in control group were administered saline injections.

Weights of animals were determined shortly before dosing, and periodically during observation period (14 days). Throughout the observation period, clinical observations were performed once a day.

The animals were sacrificed after 14 days. Liver, kidney and spleen samples were taken for histopathological examination. The samples were fixed 4% buffered formaline solution (pH = 7.4), and embedded in paraffin wax blocks after dehydration, and 2 mm sections were stained with hematoxylin and eosin.

RESULTS AND DISCUSSION

Potentiometry

The ligands and complexes were synthesized according to the reaction scheme shown in Fig. 3 (14, 15). Determination of ligand protonation constants as well as stability constants complexes with Gd^{3+} and endogenous metal ions is essential for understanding physiological tolerance of gadolinium chelates (16). Potentiometric titrations of ligands with NaOH were performed in 0.1 mol L^{-1} $NaNO_3$ solution at 20°C. The protonation constants were measured in the pH range 2.5 – 10.5. The potentiometric titration curves for ligands are shown in Fig. 4. All the titration curves of H_2L1, H_2L2, H_2L3, and H_2L4 show an increase within pH range 4.0 – 5.8, and the second increase within pH range 6.6 – 9.0.

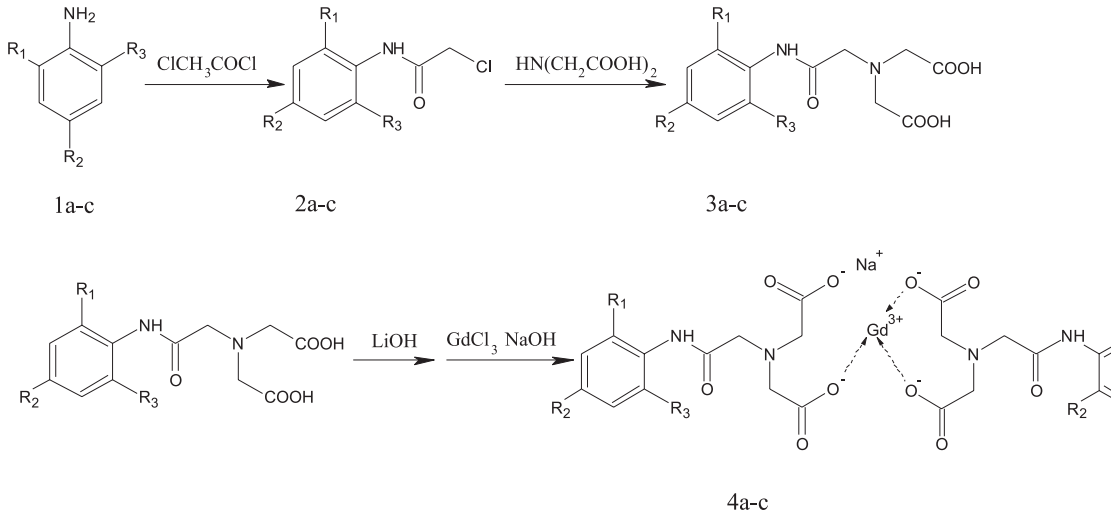

Figure 3. Synthesis and structure of 2,2'-(2-phenylamino-2-oxoethyl) iminodiacetic acid derivatives

	R_1	R_2	R_3
a	H	H	H
b	CH_3	H	CH_3
c	CH_3	CH_3	CH_3

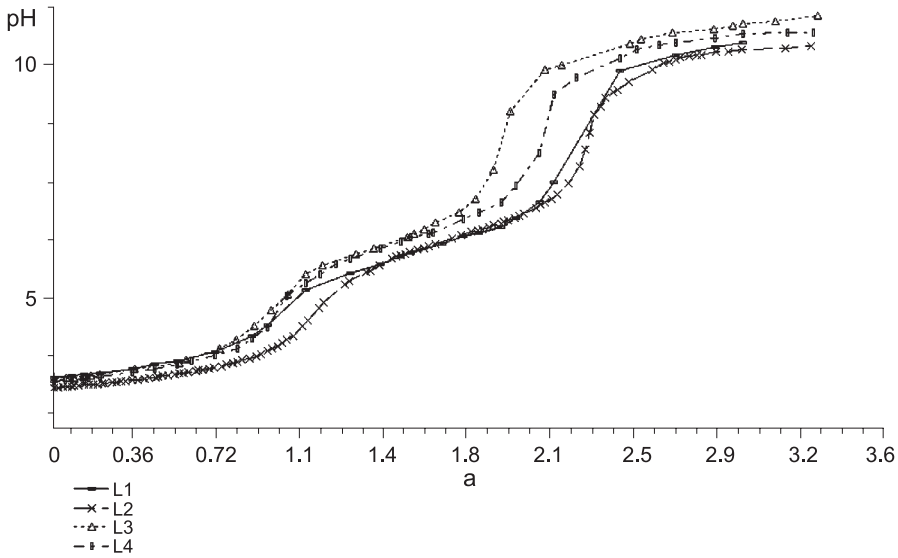

Figure 4. Potentiometric titration curves for protonation of: Li_2L1, Li_2L2, Li_2L3, and Li_2L4; a – moles of base per mole of ligands

The protonation constants of ligands (L1, L2, L3, L4), defined as

$$K_n = [H_nL] / [H_{n-1}] [H^+] \quad (1)$$

have been calculated with SUPERQUAD program (17) using titration data, and calculation results are shown in Table 1. Log K_a values of ligands are expected to be similar because of their similar chemical structures. The first protonation (with constants 6.04, 5.80, 5.65 and 6.11, respectively) presumably takes place at the nitrogen atom, the second lower values (2.07, 2.15, 2.20, 2.19, respectively) are assigned to protonation of one of the carboxylate groups. The third pK_a value for the second carboxylate group is below 2 and is not observed in the pres-

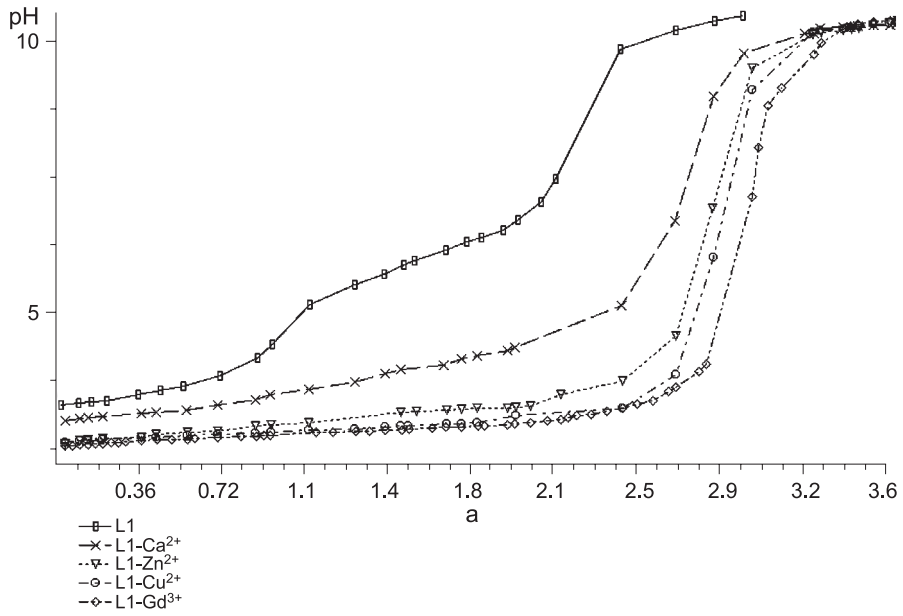

Figure 5. Potentiometric titration curves for protonation of Li_2L1 and metal salts of Ca^{2+}, Zn^{2+}, Cu^{2+} and Gd^{3+} in 1 : 2 ratio; a – moles of base per mole of ligands

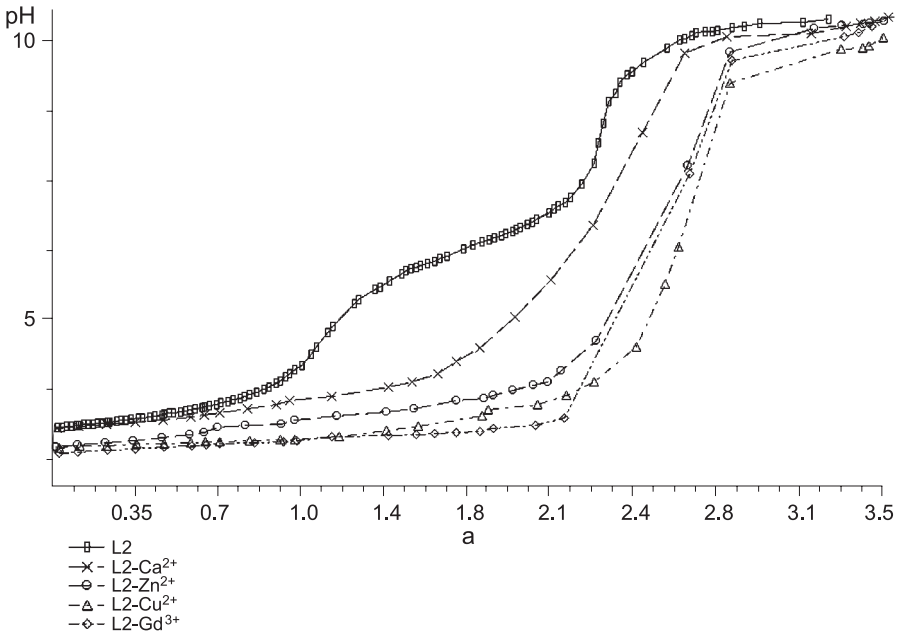

Figure 6. Potentiometric titration curves for protonation of Li_2L2 and respective metal salts in 1 : 2 ratio; a – moles of base per mole of ligands

ent study. The basicity values of ligands follow the order L4 > L1 > L2 > L3.

Stability constants of gadolinium complexes (GdL1, GdL2, GdL3, GdL4) were calculated as thermodynamic stability constants, expressed by eq. 2:

$$K_{ML(therm)} = [ML] / [M] [L] \quad (2)$$

where: [ML] = complex concentration, [M] = free metal ion concentration and [L] = concentration of free (uncomplexed, deprotonated) ligand.

The solutions containing appropriate metal and ligand in 1 : 2 ratio were titrated with standard

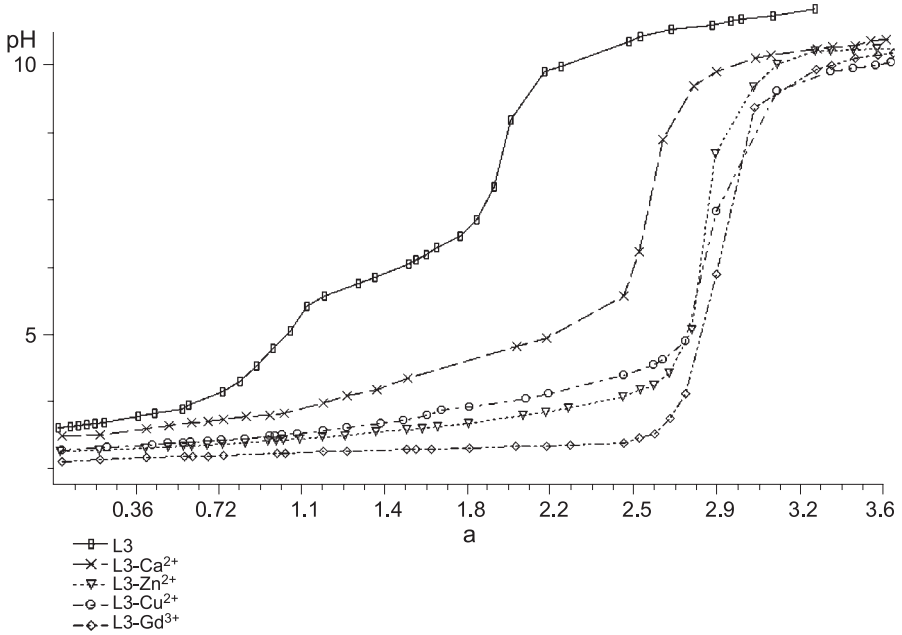

Figure 7. Potentiometric titration curves for protonation of Li_2L3 and respective metal salts in 1 : 2 ratio; a – moles of base per mole of ligands

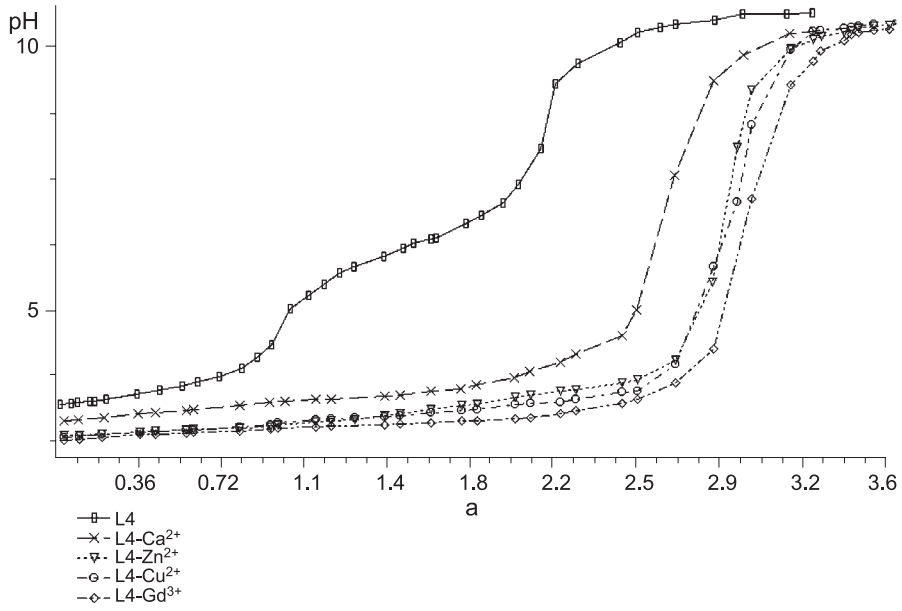

Figure 8. Potentiometric titration curves for protonation of Li_2L4 and respective metal salts in 1 : 2 ratio; a – moles of base per mole of ligands

NaOH solution, in order to determine stability constants of the complexes. The titration curves for M-L1 are shown in Fig. 5, for M-L2, in Fig. 6, for M-L3 in Fig. 7, and for M-L4 in Fig. 8. The titration data were used to calculate the stability constants of complexes with SUPERQUAD program. Table 2 contains thermodynamic stability constants of all complexes. Similar stability constants values of examined ligands may point to their similar basicity.

Conditional stability constants express stability at physiological pH (pH = 7.4) and are more important within the context of biological studies, and

Table 1. Log K_a protonation constants of ligands; 20°C, I = 0.1 mol L^{-1} ($NaNO_3$).

Equilibrium	Log K_a			
	H_2L1	H_2L2	H_2L3	H_2L4
Log $K_{1\ [HL]/[L][H]}$	6.11 (± 0.01)	5.80 (± 0.03)	5.65 (± 0.04)	6.04 (± 0.05)
Log $K_{2\ [H2L]/[HL][H]}$	2.07 (± 0.05)	2.15 (± 0.03)	2.20 (± 0.04)	2.19 (± 0.02)

Table 2. Stability constants of of Gd^{3+}, Ca^{2+}, Cu^{2+}, Zn^{2+} complexes; 20°C, I = 0.1 mol L^{-1} ($NaNO_3$).

Metal ion	Compound	Log β values (± SD) $[ML_2]/[M][L]^2$	Log β_{cond}
Gd^{2+}	L1	16.56 (± 0.24)	16.54
	L2	15.75 (± 0.18)	15.74
	L3	15.32 (± 0.14)	15.30
	L4	16.14 (± 0.16)	16.12
Ca^{2+}	L1	7.18 (± 0.12)	7.27
	L2	7.26 (± 0.09)	7.22
	L3	7.38 (± 0.11)	7.35
	L4	7.32 (± 0.10)	7.13
Zn^{2+}	L1	12.46 (± 0.22)	12.41
	L2	11.67 (± 0.20)	11.63
	L3	10.37 (± 0.14)	10.34
	L4	12.24 (± 0.18)	12.19
Cu^{2+}	L1	15.48 (± 0.18)	15.43
	L2	14.84 (± 0.12)	14.80
	L3	13.92 (± 0.13)	13.89
	L4	14.86 (± 0.11)	14.81

Table 3. Selectivity constants of Gd^{3+}, Ca^{2+}, Cu^{2+}, Zn^{2+} complexes; 20°C, I = 0.1 mol L^{-1} ($NaNO_3$).

Parameter	Log K_{sel}			
	H_2L1	H_2L2	H_2L3	H_2L4
Log K (Gd/Ca)	9.24	8.49	7.94	8.96
Log K (Gd/Zn)	4.10	4.08	4.95	3.90
Log K (Gd/Cu)	1.08	0.91	1.40	1.28
Log K_{sel}	7.06	6.89	7.39	7.23

useful in understanding *in vivo* stability. The conditional stability constants may be expressed by eq. 3:

$$K_{Mlcond} = K_{ML\ therm} / (1 + K_1[H^+] + K_1 K_2 [H^+]^2 + ... + K_1 K_2 K_n [H^+]^n) \quad (3)$$

Under physiological conditions a significant competition between proton and gadolinium ion occurs, depending on basicity of ligands (18). For this reason the conditional stability constants (Table 2) are lower than thermodynamic stability constants (8, 16).

The thermodynamic stability constants of gadolinium chelates are not the only important factors in considering their toxicity. The complexes may undergo *in vivo* transmetalation, ligand displacement, and spontaneous dissociation in reaction with proton or endogenous metal ions such as Cu^{2+}, Ca^{2+}, or Zn^{2+}. The competition between these ions and Gd^{3+} or ligand depends on relative ligand affinity to these metal ions and H^+ (2, 19).

The correlation between Gd ligand selectivity and complex toxicity may also be expressed by Gd selectivity constant (K_{sel}), which is the difference between stability constant (log K value) of Gd complex and stability constant of the complex with endogenously available metal ions. Higher selectivity value toward Gd^{3+} suggests that gadolinium ions are not displaced in the chelates by that endogenous ions. The selectivity constant for Gd^{3+} over other endogenous ions such as Cu^{2+}, Ca^{2+}, Zn^{2+} can be calculated using eq. 4:

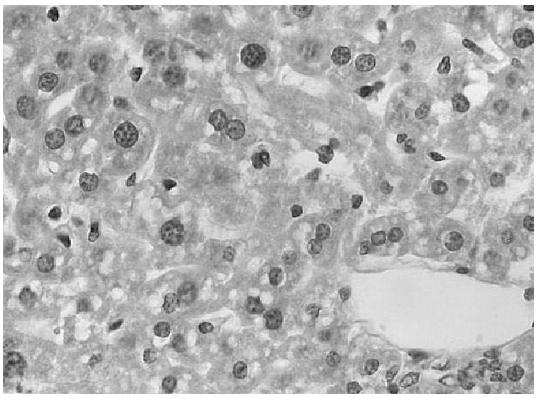

Figure 9. Small focal lymphocyte infiltration in liver sample taken from an animal which was administered gadolinium complex with sodium salt of N-(3-bromo-2,4,6-trimethylacetanilide) iminodiacetate (GdL3)

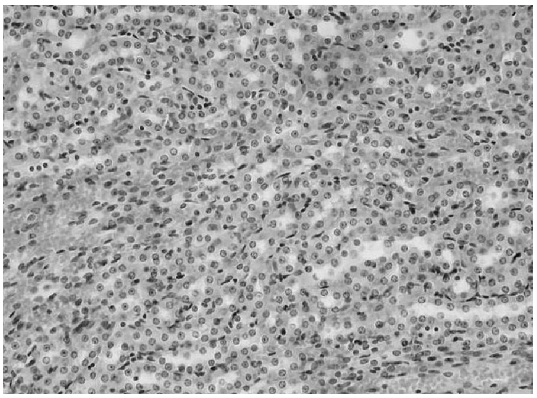

Figure 11. Kidney sample showing congestion, taken from an animal which was administered gadolinium complex with sodium salt of N-(2,4,6-trimethylacetanilide)iminodiacetate (GdL1)

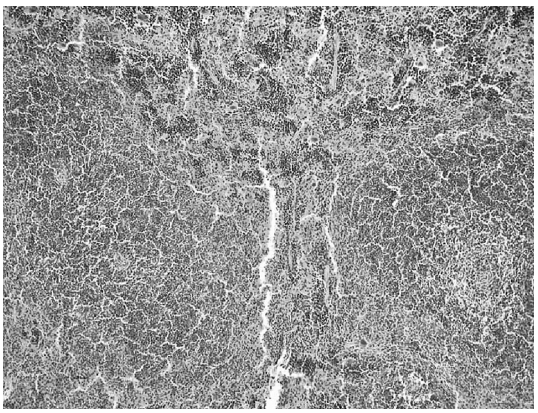

Figure 10. Spleen sample showing congestion, taken from an animal which was administered gadolinium complex with sodium salt of N-(2,4,6-trimethylacetanilide)iminodiacetate (GdL1)

$$K_{sel}' = K_{ML(therm)} (\alpha_H^{-1} + \alpha_{CaL}^{-1} + \alpha_{CuL}^{-1} + \alpha_{ZnL}^{-1})^{-1} \quad (4)$$

where α 's stand for side reaction coefficients which are defined as in reactions below:

$$\alpha_H^{-1} = 1 + K_1[H^+] + K_1K_2[H^+]^2 + K_1K_2K_3[H^+]^3 + ... \quad (5)$$

$$\alpha_{CaL}^{-1} = 1 + K_{CaL}[Ca^{2+}] \quad (6)$$

$$\alpha_{CuL}^{-1} = 1 + K_{CuL}[Cu^{2+}] \quad (7)$$

$$\alpha_{ZnL}^{-1} = 1 + K_{ZnL}[Zn^{2+}] \quad (8)$$

The concentrations of Cu^{2+}, Ca^{2+} and Zn^{2+} ions in plasma were taken from the literature as 1 µM, 2.5 µM, and 50 µM, respectively (18). The selectivity stability constants of GdL1, GdL2, GdL3 and GdL4 are given in Table 3.

GdL1, GdL2, GdL3 and GdL4 complexes under examination, feature lower thermodynamic stability constants (16.56; 15.75; 15.32; 16.14, respectively) than other gadolinium complexes applied as contrast agents, such as Gd-DTPA or Gd-DOTA with stability constants of 22.46 and 25.30, respectively (20). However, the selectivity stability constants of GdL1 (7.06), GdL2 (6.89), GdL3 (7.39) and GdL4 (7.23) are similar to selectivity constants of contrast agents Gd-DTPA (7.04) and Gd-DOTA (8.3). It may suggest that our ligands show better selectivity toward Gd than toward other metal ions, and their tolerance under physiological conditions was good.

Acute toxicity studies

Acute toxicity examinations of gadolinium complexes were performed in Swiss albino mice. Each group of eight mice was given two doses of the compound, either 200 mg/kg by intraperitoneal injection or 2000 mg/kg by oral administration. In each experiment, 10 groups of 8 animals (4 males and 4 females) were tested. The following results of the experiments were observed. In each group, all animals survived and did not display any clinical signs of toxicity, and no effects on body weight, no changes in behavior and no skin changes were noticed. After administering a dose of 2000 mg/kg *p.o.* of examined complexes no mortal cases occurred, so according to OECD guideline 423, they can be classified as non toxic. It means that LD_{50} exceeds 2 mmol/kg. The same results were obtained in the experiment with gadolinium chelates administered *i.p.* in the dose of 200 mg/kg.

Histopathological observations

Animals were sacrificed 14 days after administration of complexes. The liver, spleen and kidney samples were promptly taken and tested. Upon oral administration, the main changes in the liver included small focal necrosis of hepatocytes, small focal lymphocyte infiltration, high mitotic activity, signs of hepatocyte regeneration and congestion.

In groups of animals which were administered gadolinium complex with sodium salt of N-(3-bromo-2,4,6-trimethylacetanilide)iminodiacetic acid, changes were observed in one animal within the group (Fig. 9). In other groups, the only changes involved insignificant congestion. However, similar changes were observed in control groups. So, we suppose that the observed focal liver damage was reversible and was not caused by the administered complex.

The main changes in spleen involved insignificant congestion observed either in tested or in control groups (Fig. 10). Changes in kidneys were similar to the ones observed in spleen (Fig. 11). Following intraperitoneal injections, in none of the investigated groups of animals histopathological changes in organs were observed.

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