Toxicity and biodistribution of *para*-sulfonato-calix[4]arene in mice†‡

Anthony W. Coleman,*[a] Said Jebors,[a] Sebastien Cecillon,[a] Pascale Perret,[b] Dominique Garin,[b] Danièle Marti-Battle[b] and Marcelle Moulin[b]

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The acute *in vivo* toxicity of *para*-^{35}S-sulfonato-calix[4]arene has been determined, with no toxicity for doses up to 100 mg kg^{-1}. Biodistribution shows that the molecule is rapidly cleared in urine, that it does not accumulate in the liver and spleen, and is not observed in the brain.

It was 21 years ago that I first synthesised *para*-sulfonato-calix[4]arene in the chemistry laboratory of the University of Alabama as a postdoctoral fellow in Jerry Atwood's group, and 20 years since we first published this compound.[1] In the elapsed time, I probably inhaled considerable quantities of powdered *para*-sulfonato-calix[4]arene, and have certainly, on several occasions, accidentally injected myself with this molecule *via* cuts and other minor intrusions. My continuing life suggests that *para*-sulfonato-calix[4]arene is probably not very toxic, but there has been no proof of innocuity until now. In this article, we wish to reassure Jerry that we are, and never were, in any real danger from *para*-sulfonato-calix[4]arene (Fig. 1).

Calix[*n*]arenes are probably the most widely studied of all organic supramolecular host systems.[2] Their usage has been strongly promoted by the fact that the *para*-aromatic position and the phenolic hydroxyl groups have very widely differing chemistries, allowing chemical modification without any need for protection–deprotection strategies.[3]

Basic calix[*n*]arenes have essentially zero aqueous solubility,[2] and the first water soluble derivative, where four carboxylate groups are attached at the phenolic face, was prepared in 1984 by Ungaro *et al.*[4] However, even for this compound, its aqueous solubility is only 5×10^{-3} to 5×10^{-4} M in water, depending on the cation.

In 1984, the synthesis of *para*-sulfonato-calix[*n*]arene was reported in a communication by Shinkai *et al.*[5] In the solid state, *para*-sulfonato-calix[4]arene has a strong tendency to form sheet bilayer structures resembling clay minerals, and hence we named these organic clays.[6] Much later, I came back to the original thought of the structures resembling phospholipid bilayers, when the structure of the first bilayer-spanning complex, that with lysine, was determined.[7] Its structural diversity has been demonstrated during the past twenty years with motifs ranging from zig-zag layers,[8] through platonic and Archimedian solids,[9] spheres and tubes,[10] to Russian doll complexes.[11]

The biological activity of *para*-sulfonato-calix[4]arene and its higher analogues has proved to be quite remarkable.[12] Patents and publications have covered the use of this compound as an ion channel blocker,[13] an anti-viral,[14] a co-crystal former,[15] an anti-coagulant,[16] an amplifier for the detection of the pathogenic prion protein conformer,[17] and even as a diagnostic agent for Alzheimer's disease.[18]

Given the wide range of biomedical applications, we have devoted considerable effort to studying the interactions of *para*-sulfonato-calix[4]arene with bio-molecules.[12] Initial studies were on the complexation of amino acids, where, as expected, electrostatic interactions led to strong complexation of arginine and lysine, K_{ass} being 1500 and 740 M^{-1}, respectively.[19] The work was extended to cover di- and tri-peptides of lysine and arginine.[20]

More recently, the use of electrospray mass spectrometry has allowed the demonstration that *para*-sulfonato-calix[4]arene forms 1 : 1, 2 : 1 and 3 : 1 complexes with bovine serum albumin, the binding constants being 7.7×10^5, 3.8×10^5 and 0.3×10^5 M^{-1}, respectively.[21]

The use of *para*-sulfonato-calix[4]arene as a drug transporter has recently been studied by Lu *et al.*, who showed the solubilisation of Vitamin K_3.[22]

Given the range of possible biomedical applications of *para*-sulfonato-calix[4]arene, the next logical step is to measure the toxicity and bioavailability of *para*-sulfonato-calix[4]arene. *In vitro* studies have shown zero haemolytic toxicity for concentrations up to 5 mM.[23] A lack of non-specific immune response towards *para*-sulfonato-calix[4]arene has also been observed.[24] In an aside to a patent, *para*-sulfonato-

Fig. 1 Molecular structure of *para*-sulfonato-calix[4]arene.

[a] *IBCP CNRS Univ Lyon 1, UMR 5086, 7 passage du Vercors, 69367 Lyon, France. E-mail: aw.coleman@ibcp.fr; Fax: +33 4 7272 2690; Tel: +33 4 7272 2640*
[b] *Unité INSERM U877 (Radiopharmaceutiques Biocliniques), CHU Michallon, Hôpital de Grenoble, 38700 La Tronche, France. E-mail: Marcelle.Moulin@sim.hcuge.ch*

† Dedicated to Professor Jerry Atwood on the occasion of his 65th birthday.

‡ All experiments were performed in compliance with the relevant laws and institutional guidelines, and the Animal Experimentation Committee of CHU Michallon, Hôpital de Grenoble approved the experiments.

Table 1 Survival of mice injected with *para*-^{35}S-sulfonato-calix[4]arene

Time/min	Mouse[a]	Dose/mg	Dose/mg kg^{-1}	Survival
5	1S5C1	1	40	✓
5	2S5C1	1	40	✓
15	1S15C1	1	40	✓
15	2S15C1	1	40	✓
30	1S30C1	1	40	✓
30	2S30C1	1	40	✓
60	1S60C1	1	40	✓
60	2S60C1	1	40	✓
5	1S5C2	2.5	100	✓
5	2S5C2	2.5	100	✓
15	1S15C2	2.5	100	✓
15	2S15C2	2.5	100	✓
30	1S30C2	2.5	100	✓
30	2S30C2	2.5	100	✓
60	1S60C3	10	400	✗
60	1S60C4	5	200	✓[b]

[a] Code for the mice: First number corresponds to the individual, S for souris, second number to the time before sacrifice, C for concentration and the third number to the four different concentrations. [b] Urine contaminated with blood.

calix[4]arene was shown to have no toxicity towards various tumoral cell lines.[25]

In this Letter, we will describe the first *in vivo* studies of the biodistribution and pharmacokinetics of *para*-sulfonato-calix[4]arene with regard to mice. The methodology for the radioisotopic labelling of *para*-sulfonato-calix[4]arene will also be described.

For the radioisotope labelling of *para*-sulfonato-calix[4]-arene, it was decided to introduce ^{35}S during the final sulfonation step of the synthetic route. After numerous trials, a suitable protocol was determined.

The minimum quantities of reagents were 1 g of *para*-sulfonato-calix[4]arene and 4 mL of H_2SO_4. Strangely, a 50 mL round-bottomed flask was the minimum size for the reaction vessel, with heating for 4 hours at 90 °C. The radiolabelled *para*-^{35}S-sulfonato-calix[4]arene was isolated, after cooling to room temperature, by the addition of 1 mL of methanol, and then the addition of the resulting solution to 300 mL of ethyl acetate. After stirring for 30 min, the product was obtained by filtration and drying. In our hands, only the above protocol yielded quantitatively *para*-^{35}S-sulfonato-calix[4]arene in better than 95% purity.§ A major consequence of the above synthetic route was that, for safety reasons, we used only 100 μL of ^{35}S-H_2SO_4 of the 4 mL total. This meant that only 2.5% of the sulfonate groups were labelled. This was confirmed by the determination of the specific activity of the batch, which was 0.02 μCi M^{-1}.

For the biodistribution and initial pharmacokinetic experiments, a total of 16 mice were used. Initial doses for the intravenous injection of *para*-^{35}S-sulfonato-calix[4]arene were 100, 50, 25 and 10 g L^{-1}. The injected doses ranged from 10 mg in 100 μL to 1 mg in 100 μL, corresponding to 10 mg and 1 mg, respectively, per mouse. These values correspond to doses of 400, 200, 100 and 40 mg kg^{-1}. The acute toxicity, as a function of time, after injection is shown in Table 1, along with certain comments.

Evidently, at 400 mg kg^{-1}, the acute LD50 has largely been attained, with the only mouse tested at this concentration being dead after 60 min. In view of this, the highest injected concentration was reduced to 200 mg kg^{-1}. Here, while the dose was not lethal, the presence of blood in the urine of the mouse was observed. At doses of 40 and 100 mg kg^{-1}, there were no fatalities, the urine was not contaminated and the behaviour of the mice was normal.

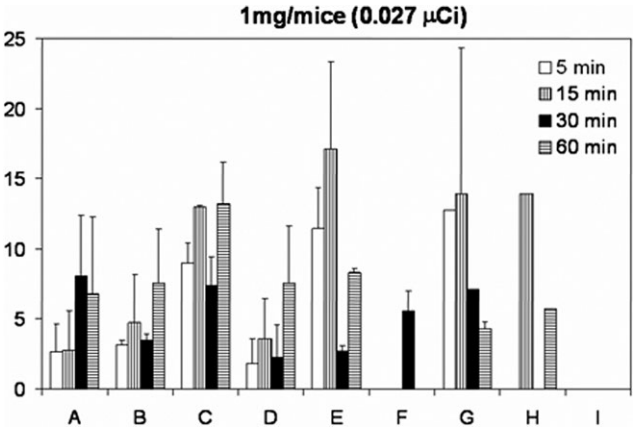

Fig. 2 Biodistribution of *para*-^{35}S-sulfonato-calix[4]arene: A = heart, B = liver, C = kidney, D = spleen, E = lungs, F = muscle, G = plasma, H = blood, I = brain at a dose of 40 mg kg^{-1}.

This translates, for average human weights (60–100 kg) to the lethal dose of *para*-^{35}S-sulfonato-calix[4]arene being between 25 and 40 g injected intravenously in a single dose. This has, of course, not been confirmed for humans. By extrapolation, injected doses in the range of 2–5 g might possibly be expected to be safe for humans. The effective concentrations for toxicity are over 100 mM, but with ionic strengths much higher than the physiological value of 0.157 M, it is certainly possible that an ionic shock is playing some role in the observed toxicity.

Fig. 2 and Fig. 3 give the biodistributions of *para*-^{35}S-sulfonato-calix[4]arene in various organs and physiological fluids at different times of sacrifice for the mice.

Table 2 shows the distributions of *para*-^{35}S-sulfonato-calix[4]arene in the urine of mice at various times prior to sacrifice.

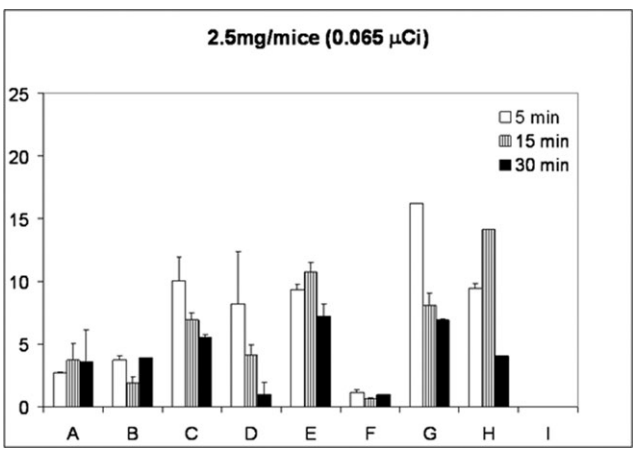

Fig. 3 Biodistribution of *para*-^{35}S-sulfonato-calix[4]arene; A = heart, B = liver, C = kidney, D = spleen, E = lungs, F = muscle, G = plasma, H = blood, I = brain at a dose of 100 mg kg^{-1}.

Table 2 Distribution of *para*-^{35}S-sulfonato-calix[4]arene in the urine of mice as a function of time, calculated as percentage of the injected dose in 50 μL of urine[a]

	Urine % DI per 50 μL	
Time/min	Dose 40 mg kg^{-1}	Dose 100 mg kg^{-1}
5	ND	35.45 ± 23.36
15	3.44 ± 2.15	26.8
30	16.6 ± 6.71	15.44 ± 6.46
60	14.77	ND

[a] DI = dose injected, ND = not determined.

A number of points are clear: *para*-^{35}S-sulfonato-calix[4]-arene does not appear to cross the blood–brain barrier or pass into muscles. This is not unexpected, as the barriers to such passage do not open to large and highly anionic, hydrophilic molecules. *Para*-^{35}S-sulfonato-calix[4]arene is rapidly cleared from the body in urine, as evidenced by its presence in urine and the kidneys. At a dose of 40 mg kg^{-1}, the amount present in blood peaks after 30 min and decreases to half of this peak value after 60 min, thus showing rapid clearance. There is no significant uptake in the organs, and particularly in the liver and spleen. Passage into muscle tissue also does not appear to occur. At higher doses, there is some accumulation in the lungs.

In conclusion, we have shown that *in vivo*, *para*-^{35}S-sulfonato-calix[4]arene shows no acute toxicity for single injected doses equivalent to 2–5 g in humans, and that the molecule is rapidly cleared from mice *via* elimination in urine. In view of the rapidity of clearance and the lack of accumulation in the liver, it is unlikely that *para*-^{35}S-sulfonato-calix[4]arene is metabolised.

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§ The experimental protocol was developed using non-radioactive H_2SO_4, and represents a method that showed full substitution and >95% purity by HPLC and ES/MS.

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