ELSEVIER

Coordination Chemistry Reviews
149 (1996) 11–22

COORDINATION CHEMISTRY REVIEWS

Aluminium toxicity and metal speciation: established data and open questions

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Received 23 January 1995

Contents

Abstract

Histopathological findings in humans and extensive toxicological investigation in vivo and in vitro point to an unambiguous neurotoxic potency of Al(III). Experimental toxicology with aqueous Al(III) is very difficult owing to the complex and somewhat uncontrollable aqueous chemistry of the metal centre in the neutral range. The choice of neutral, hydrolytically stable synthetic toxins makes possible both the control of metal speciation and of analytical metal concentration down to about 1 mM in neutral buffered solutions. The employment of less stable complexes like $Al_2(citrate)_2(H_2O)_6$ and $Al(lactate)_3$ or of ordinary salts is unavoidably complicated by the precipitation of $Al(OH)_3$ under the same conditions. In spite of this, the choice of a carefully designed protocol, based on ensuring well defined steps, enables one

SSDI 0010-8545(95)01211-7

to successfully control the analytical metal concentration down to 10 μM Al(III). The control of the metal speciation at these concentration levels remains an open question.

Keywords: Aluminium(III) toxicity; Metal speciation; Biological effects

1. Introduction

Life evolution inside an exceedingly Al(III)-rich biosphere [1] has apparently not succeeded in developing a useful biological function of this metal centre. On the contrary, compelling evidence has shown that abnormally high aluminium levels are linked to socially relevant pathologies such as dementia dialitica DD [2], iron-adequate microcytic anaemia [3], osteomalacia [4] and possibly Alzheimer's disease AD [5]. In this connection, it is convenient to stress that a causative effect of abnormal uptake of Al(III) has been established beyond any doubt only for dementia dialitica and for iron-adequate microcytic anaemia [2]. Prevention strategies carried out in modern dialysis centres have strongly reduced the occurrence of dementia dialitica [2] and experimental therapies with an effective chelating ligand have also shown some positive effects on the recovery from Alzheimer's disease [6]. However, beyond the remarkable success of the above mentioned prevention strategies, it has to be stressed that the pathogenesis of this form of encephalopathy still remains a challenge in terms of molecular toxicology.

The goal of understanding the cellular and eventually the molecular bases of aluminium toxicity has stimulated enormous experimental efforts in developing animal [7], cellular [8] and molecular models of metal toxicity [9]. Inspection of the literature reveals that experimental animals have been by far the most utilized toxicological model and a real burst of interest in this connection can be safely fixed in the late 1960s, after the appearance of two seminal papers by Wisniewski and co-workers in 1965 [10,11]. Further impetus to developing animal models of aluminium toxicity was given 10 years later by two independent and unrelated papers. In 1976 Alfrey et al. [12] provided the first convincing evidence that aluminium overload intoxication in dialyzed uraemic patients was strongly related to DD; in the same year McLachlan et al. [13] reported first that significantly elevated aluminium levels are present in selected brain areas in patients affected by AD [14]. Today there are few doubts on the presence of abnormal levels of Al(III) in AD characteristic histopathological features such as neurofibrillary aggregates and senile plaques [15].

It is convenient, at this point, to summarize some analytical, histopathological and clinical features of AD, DD and of experimental encephalopathy (EE), which are relevant to the development of this discussion. The evaluation of these articulated and interrelated results will lead the reader to appreciate the uncommon complexity of the subject, which is made even more complicate by problems related to administration protocols, dose-response control and metal speciation as pointed out in the next section.

In AD, Al(III) was found to accumulate in nuclear chromatin of cortical and hippocampal (inter alia) neurons as well as inside the abnormal protein aggregates

present in dystrophic neurons (neurofibrillary degeneration) typical of the disease [14] and in senile plaques [16]. In DD, abnormal Al(III) levels are generally distributed in all brain areas and some authors observed a particular accumulation in neuronal lysosomes [17]. Remarkably, no neurofibrillary degeneration resembling that typical of AD is observed. In EE, neurofibrillary degeneration is produced by administration of Al(III) to rabbits, cats, ferrets and dogs, but not to mice, rats, guinea pigs, hamsters and monkeys. In sensitive animals EE is accompanied by cellular localization of Al(III) similar to that observed in AD patients [7]. Most remarkably, neurofibrillary tangles were not produced in rabbits upon action of many metal centres, including In(III), Cr(III), Ga(III) and Cd(II) [18,19]. However, the experimentally induced neurofibrillary degeneration differs markedly from that observed in AD brains, both from the ultrastructural and topographical points of view [7]. However, in spite of this, in both AD and EE, hippocampus is the brain region most affected by neurofibrillary changes! To make the story even more complicated, Al(III)-associated senile plaques are observed in AD brains, but not in EE and, controversially, in DD [20].

In this introductory section it is worth briefly mentioning and underlining some very fine analytical, histopathological and clinical observations in experiments on rabbits [7]. Thus, after Al(III) administration, it was possible to separate in the time scale relevant toxic consequences: (i) metal accumulation in the animal brains occurs after hours; (ii) neurofibrillary degeneration after few days; (iii) behavioural deficits, including loss of memory, after 8–10 days. These observations point to two suggestive molecular hypotheses for EE, i.e. the metal centre may produce multiple alterations in the transcription of genetic information [13–21] and/or may induce abnormal protein synthesis.

2. Methodological problems associated with Al(III) experimental pathology

The wealth of toxicological information available in the literature is potentially affected by a fundamental methodological problem (and consequently by a serious bias), i.e. the very scarce solubility of Al(III) at physiological pH values and the ill-defined nature of the Al(III)–H_2O–OH^- system under practical operational conditions (Fig. 1). Apart from a very few recent studies (see below), toxicological work has been carried out by starting from a variety of Al(III) salts or complexes in which the counter ion was either the conjugated base of a strong acid (Cl^-, SO_4^{2-}, NO_3^-) (type I) or of weak α-hydroxocarboxylic acids (lactate, citrate, tartrate) (type II). Type I salts undergo extensive hydrolysis in water giving rise to acidic solutions which, after neutralization, do unavoidably produce $Al(OH)_3$. The same occurs for type II toxin, when analytical concentrations are in the millimolar range [23] (see below).

Amorphous $Al(OH)_3$ is in itself a rather ill-defined material [24], whose solubility decreases with aging. Owing to the well known kinetic inertness of Al(III) complexes in neutral solutions [25,26], the molecular composition (vide infra) of the mixture of aquo-hydroxocomplexes is predicted to be out of equilibrium with $Al(OH)_3$ and is likely to change with time. Upon neglecting this very important circumstance,

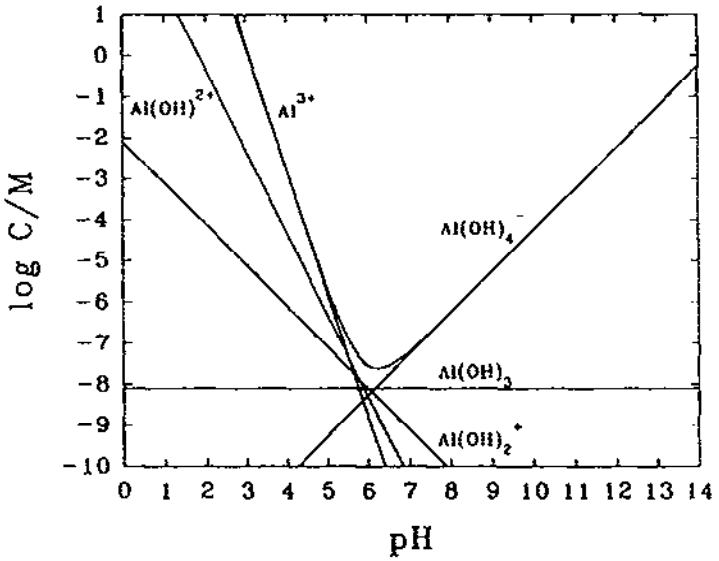

Fig. 1. Solution state of Al^{III} in the absence of strongly coordinating agents (see text), as predicted from thermodynamic data (23). In the formulas, metal-coordinated water molecules are omitted.

thermodynamics predict that the dominating species at pH 7.5 is $(Al(OH)_4)^-$ with a predicted concentration of about 0.1 mM.

In view of the traditional protocols utilized in toxicological work by research in the past decades (see Section 3) we can safely observe that the great majority of the biological results reported in the literature refer to the actual use of aqueous suspensions of $Al(OH)_3$. The obvious consequences of this conclusion are the following:

(a) the qualitative composition (speciation) of the Al(III)-containing aqueous suspensions may have been out of control, on the basis of trivial time-related circumstances;

(b) the total amount of Al(III) (dose) believed to be administered to the biological system may also have been out of control, owing to the presence of most of the metal centre as a fine, often unobservable, precipitate.

On the basis of the above, a diffuse lack of speciation and dose control in experimental pathology is most likely to have occurred. This circumstance might well explain quite a number of contradictory results reported in the literature and clearly pointed out in Ref. [27], especially in connection with delicate investigations regarding enzymatic activities and Al(III)-promoted development of synaptic effects.

3. Common experimental protocols utilized in experimental toxicology in vivo and in vitro

3.1. Toxicology in vivo

The administration routes utilized in animal experimentation have been most diverse and are listed in Table 1, together with some technical details.

Table 1
Essential information on common protocols utilized in toxicological in vivo experimentation

Protocol	Technical notes	Selected references
Oral	the toxicant is left to be ingested by various means	[28]
Intravenous	through a vein directly into the circulatory system	[29]
Intraperitoneal	within the peritoneum cavity	[30]
Intracranial	a toxicant solution is injected into brain ventricles	[31]
Subcutaneous	a toxicant solution is injected beneath the skin	[32]

It has to be noted that in the majority of the papers examined, details on the preparation of solutions or suspensions to be administered have been very scanty, sometimes non-existent. Often the commercial source or the synthesis of the aluminium compounds and their purity levels were not reported. Rather frequently the pH value of the administered solutions is not reported, so that it can be reasonably expected that in some cases acidic Al(III) solutions have been administered tout court. When the solutions to be administered were neutralized (or directly prepared in a convenient buffer) we very rarely noticed $Al(OH)_3$ precipitation. The time passed between the preparation of the neutralized suspensions and their administration appears to have been regularly ignored.

3.2. Toxicology in vitro (cell cultures)

A typical experiment is performed by seeding cell cultures in Petri dishes in the presence of a solution containing essential nutrients, buffer, calf serum and the relevant toxicant (see for example Ref. [33]). In the very large majority of the reported cases, the same observations put forward above are also valid in this kind of experimentation and a similar unawareness is apparent in the relevant literature.

4. Metal speciation and differential biological effects

As already noted above, toxicologists have utilized a variety of metal salts or complexes for preparing their 'solutions' to be utilized both in vivo and in vitro. Although the feeling can be perceived in some papers that speciation effects may be occurring, clear awareness in this connection appears to be evident only in very recent papers (see for example Refs. [34] and [35]). Consequently, very few studies are available in which a given biological system is investigated on the basis of chemically (molecularly) different aluminium toxins, able to maintain their chemical identity under identical administration protocols.

As to the in vitro (cell cultures) toxicology, a recent short review is available [36] and the essential data reported therein are schematically summarized in Table 2.

Speciation effects in in vivo toxicology have been clearly observed in our laboratories since 1989, both in rabbits [41] and in rats [42]. Thus, $Al(acac)_3$ (acac=

Table 2
Speciation effects observed in Al^{III} toxicology *in vitro*

Cell system	Toxin	Effect	References
Erythrocytes (rabbit)	$Al(acac)_3$	metal uptake echinoachantocytosis	[37]
	$Al(lact)_3$	none	[37]
Neuroblastoma cells (murine)	$Al(acac)_3$	cytotoxicity	[33]
		cytotoxicity	[33]
	$Al(lact)_3$	cytostaticity with neuritogenicity metal uptake inhibition of haemoglobin synthesis	[33]
Limphocytes (human)	Al–Tf		[38]
	Al-citrate	smaller effects	[38]
Hepatocytes (mouse)	Al–Tf	metal uptake; cytostaticity	[39]
	Al-citrate	smaller metal uptake; no cytostaticity	[39]
Neurons (rat)	$Al(malt)_3$	neurofibrillary degeneration (tangles)	[40]
	$Al(OH)_3$	none	[40]

2,4-pentanedionate) (lipophilic) was found to be at least 100 times more cardiotoxic than $Al(lact)_3$ (lact = 2-hydroxypropanoate) to rabbits upon intravenous administration (total doses: 0.3 mg per kg body weight). Most remarkably, $Al(malt)_3$ (malt = 3-hydroxy-2-methyl-4H-pyran-4-onate) [43], a neutral hydrophilic complex structurally related to $Al(acac)_3$ (Fig. 2) and similarly hydrolytically stable has no effect

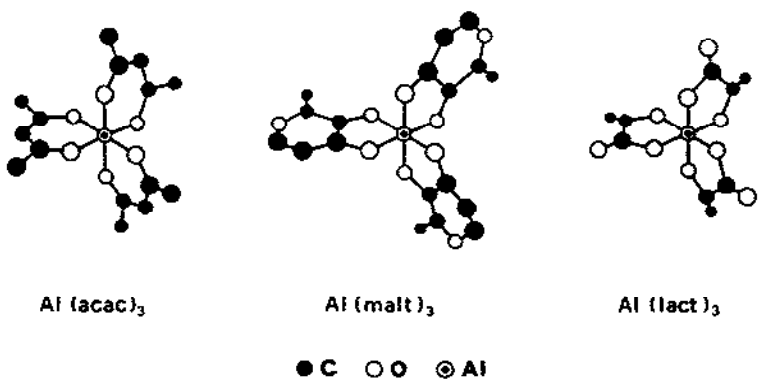

Fig. 2. Molecular structures of Al(III) synthetic toxins. Hydrogen atoms are omitted. For hydrolytic stability and lipophilicity, see Ref. [9]. (From Ref. [9], with permission.)

under identical conditions [34]. At higher doses (total doses: about 60 mg per kg body weight in 30 weeks), $Al(malt)_3$ is seen to be neurotoxic [44].

An interesting speciation effect based on physical rather than chemical circumstances is described in Refs. [45] and [46]. In fact, the cardiotoxicity to the rabbit of neutral solutions of $Al(lact)_3$ increases by two orders of magnitude if the metal centre is carried inside dipalmitoylphosphatydylcholine liposomes but, remarkably, the effect is not infarctual in character, which is the case upon injection of neutral water solutions of $Al(acac)_3$. Interestingly however, a spinal cord infarct is produced.

Another remarkable speciation effect was observed when neutral solutions of $Al(acac)_3$, $Al(lact)_3$ and $Al(malt)_3$ were injected into rats treated with ^{14}C-labelled sucrose. The experiments were aimed at confirming and extending previous reports on the ability of Al(III) to increase the permeability of the blood–brain barrier of the rat [47]. In fact, $Al(acac)_3$ and $Al(malt)_3$ were respectively found to increase irreversibly and reversibly the blood brain barrier permeability, while neutral solutions of $Al(lact)_3$ were ineffective under identical conditions [48].

Finally, it has been reported [35] that when aqueous solutions of $Al(malt)_3$ were injected intracranially to rabbits (13 μmol), it was found to be 20 times more active than $Al(lact)_3$ in inducing a lethal encephalopathy.

5. Metal speciation in neutral solutions at lower metal concentrations: experimental implications

The aim of this section is to present and discuss an aspect of the aqueous chemistry of Al(III) compounds which is the real 'crux of the problem' for developing a new-generation aluminium toxicology. Parts of these arguments have already been presented in Refs. [34] and [9].

The equilibrium speciation of a given complex AlL_6 (L = individual ligating site of mono- or polydentate ligands) in water at pH ≈ 7 depends on: (i) its hydrolytic stability (as expressed by the relevant conditional stability constant); (ii) its analytical concentration. Now consider the complex $Al(malt)_3$. The log of its conditional stability constant at pH 7.4 is 22.5 at 25 °C. In relatively concentrated solutions (e.g. 10 mM) the species $Al(malt)_3$ is the actual dominating Al(III)-containing molecular entity in solution in the 4–10 pH range (Fig. 3).

It is apparent that the predicted stability window becomes narrower for a 1 mM solution and, for a 0.1 mM solution only 50% of the complex should survive in the 6–8 pH range. At 10 mM $Al(malt)_3$, the complex is predicted to be fully dissociated to give $Al(OH)_3$ and maltol.

On the basis of the above, it is clear that the toxin concentration in the solution actually administered to the biological system or the actual concentration achieved in the culture medium (Section 3) is fundamental for defining the metal speciation at the start of the experiment.

However, kinetics may intervene and appreciably modify the thermodynamic prediction. We have performed some experiments aimed at controlling the hydrolytic fate of $Al(acac)_3$ and $Al(malt)_3$ at pH at 25 °C (Fig. 4).

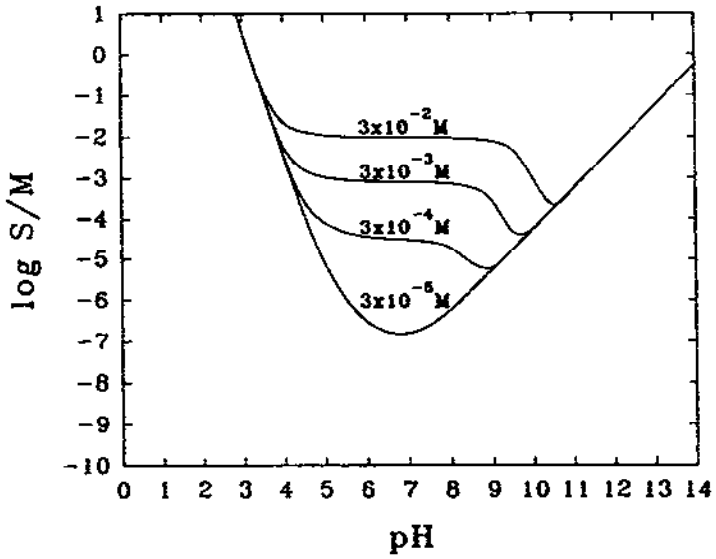

Fig. 3. Solubility of Al(III) in the presence of maltolate as the function of ligand analytical concentration and of pH; T = 25 °C. Effect of Al(III) analytical concentration on the "stability window" of $Al(malt)_3$. (From Ref. [9], with permission.)

The kinetic plot for $Al(acac)_3$ hydrolysis reveals that in a 100 μM solution about 50% of the metallo-organic ring (the chromophore whose fate the experiment is actually monitoring) is still present after about 100 h. The same plot for $Al(malt)_3$ reveals a much higher (meta)stability of this toxin both at 100 and 50 μM analytical concentration, after the same time. Therefore, in order to maintain the initial metal speciation in a toxicological experiment at the start of the experiment, $Al(malt)_3$ is better than $Al(acac)_3$ for lower toxin concentrations. If the toxicologist wishes to use a lipophilic species, $Al(acac)_3$ can be used, but with the limits outlined above.

6. The need of a general toxicological protocol able to provide control of metal speciation and/or of metal concentration: a proposal

To conclude the presentation of both the phenomenology and the reasoning of this paper, we propose a relevant protocol for aluminium toxicology in vivo and in vitro as well as for enzymological experimentation. The experimental details will be published elsewhere [49] and we anticipate that this protocol will at least solve the question of the dose control and also perhaps create the basis for the speciation control at the start of the actual toxicological experiment. Beyond the specific relevance to aluminium toxicology, we do think that this protocol will also be useful for studies centred on the utilization of compounds of metal centres prone to hydrolytic transformations at near neutral pH values, such as e.g. Cr(III) and Fe(III).

The protocol developed in our laboratories is based on the utilization of well

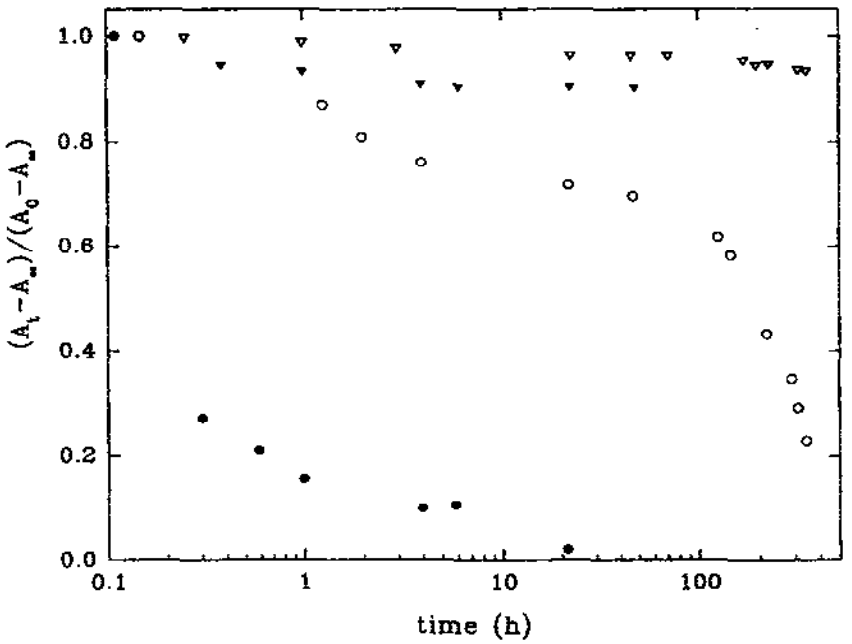

Fig. 4. Spectrophotometric evidences for $Al(acac)_3$ and of $Al(malt)_3$ (meta)stability in water at pH ≈ 7 at 25 °C. A_0, A_t and A_∞ indicate the optical density at $t = 0$, t and after complete hydrolysis at 340 nm, for $Al(acac)_3$ 10^{-4} M (○) and 5×10^{-5} M (●), and at 280 nm for $Al(malt)_3$ 10^{-4} M (▽) and 5×10^{-5} M (▼) (cell path = 1 cm).

defined aluminium compounds of known high purity. To this end we chose $AlCl_3 \cdot 6H_2O$ (Fluka) **1**, Al(lactate)$_3$ (ICN, K & K Laboratories) **2**, Al_2(citrate)$_2(H_2O)_6$ **3** [50], $Al(acac)_3$ **4** and $Al(malt)_3$ **5**. Apart from **1**, the purity of the other chemicals was checked by elemental analysis.

Weighed amounts of toxins **1**–**3** were employed for preparing 0.1 M solutions at the autogenous pH values. From these solutions, nominally 1 mM Al(III) solutions were prepared upon diluting the concentrated solutions in 0.1 M TRIS-HCl buffered solutions (pH = 7.4) at room temperature. Precipitation of aluminium hydroxide was observed and, after given times, the suspensions were centrifuged and the supernatant solutions were filtered through a 0.22 μm membrane (Sartorius) and immediately analysed. In the case of **4** and **5**, 1 mM solutions were prepared by dissolving exact known amounts of the complexes in 0.1 M TRIS-HCl buffered solutions, at room temperature. Again, after given times, the clean solutions so obtained were filtered with 0.22 μm membranes and immediately analysed.

Al(III) total concentration of the filtered solutions was determined by a standard colorimetric method [51] (**1**–**5**) and by atomic flame absorption spectrometry (**4**, **5**) for the sake of extra control.

The data referring to the nominally 1 mM solutions stored for 72 h and analysed at given times are collected in Fig. 5.

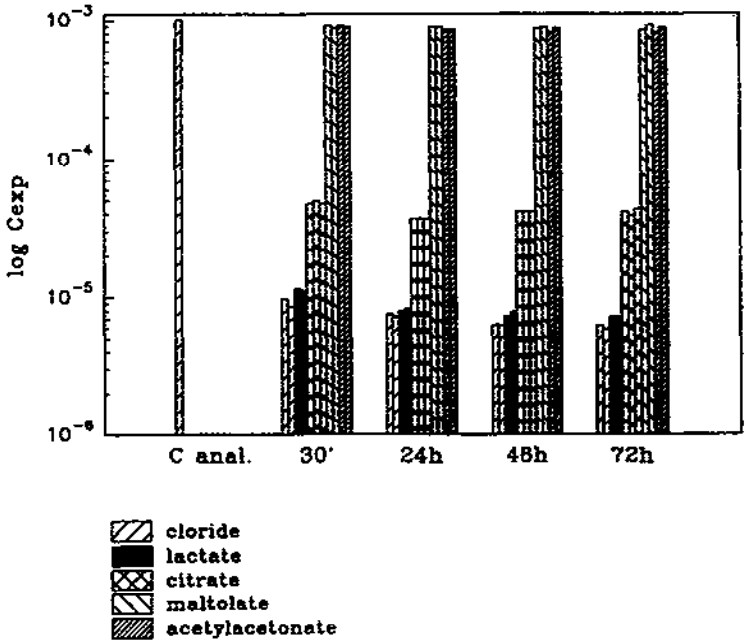

Fig. 5. Al^{III} concentrations measured after given times in nominally 1 mM solutions obtained by dissolution of known chemicals, after centrifugation (when necessary) and filtration (see text). The left bar indicates the expected metal concentration in the absence of hydrolysis. Analytical results are given in duplicate (in triplicate for aluminium citrate).

Monitoring of the metal concentration from 0.5 to 72 h revealed a substantial constancy of dissolved Al(III) concentration, in this rather large and experimentally convenient time-range.

For toxins **4** and **5** an evaluation of metal speciation at lower analytical concentration was attempted on the basis of ^1H FT-NMR spectrometry (200 MHz), in 0.1 M TRIS-DCl deuterium oxide (pD = 7.4) at room temperature. For both toxins we took advantage of the sharp signal given by the methyl resonances of the ligands involved in the metallo-organic rings.

This approach appears to be rather promising in that the survival of the metallo-organic ring has been unambiguously proven down to at least 200 μM (analytical) solutions for both toxins. This result makes reasonable the expectation to control (and check) metal speciation under really toxicologically relevant conditions [40].

7. Open questions

The careful control of metal concentration and metal speciation in directing the biological activity of Al(III) is clearly of major importance for reliable toxicological experimentation. A convenient control of metal concentration (dose) appears to be

possible on the basis of our (or a similar) protocol. Metal speciation control and detection is an open question which still has to be effectively addressed. Although our preliminary results appear very promising, molecular control down to 100 μM or less under real in vivo and in vitro conditions appears to be a formidable task. Strongly hydrolytically stable artificial toxins are to be discovered and tested, together with the setting up of spectrometric protocols most likely based on high-field NMR and FTIR spectrometries. UV spectrophotometry, albeit very sensitive (see **5**) is not sufficiently selective, under actual toxicological conditions, to be successfully utilized in this context. It may be worth mentioning, in this connection, the recent results by Sadler and co-workers [52] who showed by spin echo NMR that 100 μM aluminium citrate can be unambiguously detected in human blood.

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