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Review

The speciation of vanadium in human serum

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ABSTRACT

A knowledge of the speciation of vanadium in human serum is essential for an understanding of the biotransformation of antidiabetic vanadium complexes in human blood and of how vanadium is transported to the target cells. Such information may be acquired by two completely different approaches: separation techniques and modeling calculations. This review focuses on the latter.

The two major metal ion binders in human serum are apotransferrin (apoTf) and human serum albumin (HSA), the interactions of which with $V^{IV}O$ and V^{V} are discussed in detail. A partially new model for HSA–$V^{IV}O$ interactions is introduced, in which the two binding sites (one for two and one for one metal ion) compete not only with each other, but also with hydrolysis of the metal ion.

Focus is also placed on the possibility and importance of ternary complex formation between $V^{IV}O$, serum proteins and drug candidate ligands (maltol (mal), 1,2-dimethyl-3-hydroxy-4(1H)-pyridinone (dhp), acetylacetone (acac) and picolinic acid, (pic)): the structures and formation constants of different ternary complexes reported by the different research groups are critically reviewed.

The serum speciations for $V^{IV}O$ and V^{V} are calculated through use of the most recent stability constants; at biologically relevant concentrations (~1 μM, but definitely <10 μM) the apoTf complexes predominate for both metal ions. This has the consequences that the primary role of the drug candidate ligands of the original complexes is a carrier function until the vanadium is taken up into the serum, and the vanadium ion itself is the active metabolite responsible for the antidiabetic effect.

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1. Introduction

Vanadium compounds were first considered as potential oral drugs for patients with *diabetes mellitus* more than 100 years ago, but more intensive studies started only in the mid-1960s [1]. The first *in vivo* experiments with $NaVO_3$ were reported in 1985 [2]. In consequence of the disadvantages of inorganic salts ($NaVO_3$: gastrointestinal distress; $VOSO_4$: low absorptive efficiency) [3], mostly neutral bis complexes of $V^{IV}O$ with bidentate organic ligands (e.g. mal [4,5], pic [6] and acac [7]) have been studied; chelation of the metal ion improved its biodistribution and tolerability [1].

The Phase I clinical trial [1] of bis(ethylmaltolato) oxovanadium(IV) (BEOV) was completed in 2000, and the results of the Phase IIa clinical trial were first published in 2009 [8]. Among the many aspects of this field, focus was placed primarily on the possible biotransformation of the drug candidate vanadium compounds in the body, from the gastrointestinal tract to the target cells [9]. Most of the drug candidate compounds are not stable in the gastric juice, where the pH is ~2, and other biomolecules present in the stomach or intestines may also change the charge of the complex unfavourably. Very similarly to BEOV, ~70% of the orally administered $V^{IV}O(mal)_2$ is not absorbed, and the need for $V^{IV}O$ compounds with better absorption properties still exists, although the uptake efficacy can be tuned by means of drug formulation (e.g. via the encapsulation technique [10]). After uptake, orally administered compounds reach the blood circulation system, where they interact with the serum constituents. As concerns the speciation of vanadium in the serum, all possible biotransformations should be taken into account. We earlier concluded [9] that the strongest $V^{IV}O$ binder in human serum, apotransferrin displaces the original carrier ligands and transports the vanadium to the target cells. However, recent results relating the $V^{IV}O$–HSA interaction [11] and ternary complex formation between the drug candidate complexes with apoTf [12,13] or HSA [13] inspired us to review the importance of the possible interactions.

This paper further discusses the advantages and disadvantages of model calculations, surveys the available knowledge concerning the interactions between $V^{IV}O$ and its drug candidate complexes with apoTf, and critically reviews the sometimes inconsistent results published on the HSA–$V^{IV}O$ complexes.

2. Speciation study

The simplest speciation study involves detection of the different chemical forms of vanadium by an efficient spectroscopic tool. However, in the therapeutically important concentration range, most of the methods which can be applied without transformation of the sample (e.g. NMR, EPR and CD spectroscopy) are not sufficiently powerful to furnish details regarding the speciation. Two solutions exist: separation techniques and modeling calculations. Both can yield important information, but the latter are preferred as they can handle systems involving fast dynamic processes. Modeling calculations demand reliable information concerning stability constants and concentrations. The concentrations of vanadium (and the drug candidate ligands) are crucial [14], and data from studies performed under therapeutically irrelevant conditions can give misleading information. The number of serum components present is too high to allow determination of the stability constants of all the complexes formed with $V^{IV}O$, and pre-selection is necessary on the basis of the metal ion-binding abilities. The Harris serum model [15], devised originally for Zn^{II}, was used as a starting point. Four components for $V^{IV}O$ were identified as potential primary binders: apoTf, HSA, citrate (cit), lactate (lac), other two phosphate and oxalate (ox) can have a role only in ternary complex formation. Glycine (gly) is not considered to be relevant $V^{IV}O$ ion binders under the conditions prevailing in the serum. Histidine (his) was not taken into account either, because it does not form strong enough binary $V^{IV}O$ complexes [16], and there is no quantitative information about its ternary complex formation capability.

3. Oxidation state of vanadium in blood serum

From a therapeutical aspect, it is not essential to know the oxidation state of vanadium transported by the blood serum; although a knowledge of the form of the metal ion available for the target cells is crucial for an understanding of the biodistribution of vanadium.

The oxidation states $V^{IV}O$ and V^{V} are both relevant as concerns the biochemistry of vanadium in the blood, but the reducing agent content (e.g. ascorbic acid [17] and cysteine [18]) is theoretically adequate to effective reduction of V^{V} to $V^{IV}O$.

When V^{V} was added to fresh serum under N_2, full reduction of V^{V} to $V^{IV}O$ was observed [19]; further reduction to V(III) was not detected. However, when O_2 molecules were not excluded from the $V^{IV}O$-blood system, the reverse redox reaction, the oxidation of the $V^{IV}O$ to V^{V}, occurred [19]. Using BCM-EPR (BCM: blood circulating method), Sakurai et al. demonstrated that the EPR signal of $V^{IV}O$ disappeared quite quickly from the blood of living rats. Whereas the $V^{IV}O$ remained stable during a long period in freshly taken blood, indicating the distribution of $V^{IV}O$ but not its oxidation to V^{V} [20].

4. The concentration of the vanadium in blood serum

In the serum of a healthy person who has not been treated with vanadium compounds, the concentration of vanadium generally lies in the range 0.45–18.4 nM [21].

The maximum daily oral dose in the Phase I clinical trial was 95 mg BMOV, equivalent to 15 mg V and 0.22 mg V/kg for a 70 kg person) [1]. For an absorption efficacy of 30% [22] and an overall blood content of 5 l, if all of this vanadium enter the blood at the same time, the maximum concentration attainable would be ~20 μM. Naturally, this is only a rough estimate, but it clearly shows a well-defined limit.

In animal studies involving much higher doses up to 12 mg V/kg, two independent research groups determined the maximum vanadium concentration in the blood to be 2–3 μg/ml, i.e. ~40–60 μM. As the concentration that can be achieved increases with the dose applied [8], if a linear relationship is presumed, the highest attainable concentration for test animals in the event of a daily oral dose of 0.22 mg V/kg would be only ~1 μM. Since the maximum value of the vanadium concentration in the human blood during treatment was not published, this is the best available estimation up to now.

5. $V^{IV}O$ complexes – binary systems and interaction with low molecular mass serum constituents

The speciations of six drug candidate $V^{IV}O$ complexes ($V^{IV}O(mal)_2$, $V^{IV}O(pic)_2$, $V^{IV}O(Me\text{-}pic)_2$ (Me-pic: 6-methylpicolinic acid), $V^{IV}O(dhp)_2$, $V^{IV}O(hpno)_2$ and $V^{IV}O(mpno)_2$ (hpno: 2-hydroxypyridine-N-oxide, mpno: 2-mercaptopyridine-N-oxide)) and their interactions with four serum constituents (cit, lac, phosphate and ox) as potential binders have been studied exhaustively [23–29]. At the 100 μM $V^{IV}O$ level, when the concentration of apoTf in the serum is not enough to bind all of the $V^{IV}O$, ternary complexes of the type VOAB are formed mainly with cit and in some cases with lac and phosphate, where A is one of the original ligands and B is a constituent of the serum. At the same $V^{IV}O$ concentration level, calculations reveal the presence of small amounts (<5%) of biscitrato–$V^{IV}O$ complexes [28].

6. $V^{IV}O$ interaction with apoTransferrin

Chasteen et al. confirmed that $V^{IV}O$ binds at iron-binding sites, but similarly to Fe^{III}, only in the presence of HCO_3^- [30]. At both the C- and N-terminal sites of the protein, two O atoms of the synergistic CO_3^{2-} anion, two tyrosine-O^- atoms an aspartic carboxylate and a histidine-N atom coordinate to Fe^{III} [31], However, the X-band EPR of $V^{IV}O$–apoTf complexes indicates two different (A and B) coordination environments for $V^{IV}O$. Type A spectra are observed exclusively at pH ~6, at which apoTf is able to bind only one $V^{IV}O$ [30]. If the pH is increased to 7–8, the intensity ratio between the type A and B spectra becomes one, and the $V^{IV}O$-binding capacity increases to two. Elevation of the pH to 9 or above results in conversion of the type A environment to type B, but without change in the binding capacity [30,32]. The binding mode described by the type B spectrum is not uniform: the more powerful Q-band EPR differentiates type B_1 and B_2 coordination environments [33]. The A and B conformational states are believed to relate to the C-terminal and N-terminal binding sites of the protein, respectively [30,31,34]. Coordination of the histidine-N atom and CO_3^{2-} has been demonstrated by ESEEM experiments [35], and that of two phenolate groups of tyrosine residues has been demonstrated by UV difference spectroscopy [36].

Use of the pulse technique ENDOR (not with human apoTf, but with horse spleen apoferritin) led Aisen et al. to conclude that two N atoms, most probably both from the coordinated histidine moiety, are present in the environment of $V^{IV}O$. The replacement of H_2O by D_2O causes a reduction in intensity of the ^1H matrix ENDOR line, which means that the paramagnetic metal center is accessible to the solvent (H_2O) and most probably to the molecular O_2 too [37]. This is in agreement with the observation that the $V^{IV}O$ complexes of apoTf are quite sensitive to oxidation; independently of the A or B environment, the half-life of the $V^{IV}O$ is ~8 min under normal conditions in air [19]. Interestingly, the apoTf complexes of V^{III} are not air-sensitive [38]; Fe^{III} and V^{III} (probably with the same binding environment) ions do not seem to be accessible to H_2O and O_2. This suggests that even $V^{IV}O$ coordinates at the same binding site as Fe^{III} and the coordination of his-N, 2 tyr-O^- and the CO_3^{2-} is proven, though the exact coordination environment may be slightly different, or at least more dynamic. The explanation is plausible: the oxido-O atom of the $V^{IV}O$ unit needs space, and this to some extent inhibits the formation of the same coordinating environment as in the case of Fe^{III}.

EPR investigation of the interaction of $[V^{IV}O(mal)_2]^-$ with apoTf [39,40] clearly demonstrates complete decomposition of the maltolato complex. In order to be able to predict which $V^{IV}O$ compound will decompose in the presence of apoTf, reliable stability constants of its $V^{IV}O$ complexes are needed. Initially an estimation based on a linear free energy relationship [31] was applied [25] by our group (see Table 1); later a method involving ligand competition with NTA (nitrilotriacetic acid) yielded a very similar result [41]. The same method, but with the ligand dhp and with UV, LNT (liquid nitrogen temperature) and RT EPR for detection, first gave somewhat higher stability constants with relatively high uncertainty, the $\log\beta_1$ lying in the range 14.0–15.0 [41]. Combined CD and RT-EPR measurements clearly revealed ternary complex formation in the $V^{IV}O$–dhp–apoTf and $V^{IV}O$–mal–apoTf systems [12]. When this is taken into account, the determined stability constants are practically the same as with the other two methods (see Table 1). With EPR at −150 °C Sanna et al. determined analogous stability constants by the same method for the ligand acac; the results differed only slightly [13]. The fact that the results from different laboratories agree increases their reliability, but it must be mentioned that completely different values ($\log\beta_C$ = 5.23 for the C-lobe and $\log\beta_N$ = 4.84 for the N-lobe) emerged from a calorimetric, DSC method involving scanning from 5 °C to 100 °C [42]. The meaning of K_{app} in this latter publication and how it can relate to the data in Table 1 are not completely clear; it is possible that they are simply not comparable directly with each other.

7. $V^{IV}O$–apoTransferrin ternary complexes

Ternary complex formation in the systems $V^{IV}O$–apoTf–mal or dhp has been confirmed by joint evaluation of the EPR and CD spectra [12]. Both lobes of apoTf are able to bind a $V^{IV}O$(mal) or a $V^{IV}O$(dhp) unit, but their RT EPR spectra can be described by one EPR parameter set, which means that the coordination environment is more uniform than in the simple binary complexes of $V^{IV}O$ with apoTf. The nearly statistical difference between the calculated $\log K_1$ and $\log K_2$ values is also in accord with the similarity of the two binding sites [12]. Maltol forms less stable ternary species than those of dhp; similarly, $V^{IV}O(mal)_2$ is less stable than $V^{IV}O(dhp)_2$ [12].

Chasteen et al. determined that in the absence of HCO_3^- several (but not all) ligands containing a carboxylate function(s) are able to behave as synergistic anions. They can be classified into three groups: non-synergistic anions (e.g. acetate, succinate and glycine), synergistic anions, with only the B conformation (a weakly acidic proximal functional group, e.g. lac), which means that only one $V^{IV}O$ is bound to apoTf in the presence of the ligand, and synergistic anions for both conformations (two or more carboxylate groups is the common characteristic, e.g. oxalate) [43]. The initial idea was that dhp or mal can substitute the synergistic anion, but ternary complex formation (with dhp) was found to be non-competitive with HCO_3^-: in the absence of the synergistic anion, ternary complex formation was less favoured. In fact, a quaternary species: $V^{IV}O$–apoTf–HCO_3^-–dhp has been found [12]. This type of complex, in which the low molecular mass ligands mal and dhp are directly bound to the apoTf, cannot be imagined without a dramatic change in the $V^{IV}O$ coordination sphere. We consider that the conformation of the protein in the $V^{IV}O$ complexes of apoTf differs somewhat from that in holoTf; the former is more open, which could reflect the different and convertible coordination environment, the O_2 and water accessibility and the extra space demand of the $V^{IV}O$-oxido–O. In the quaternary complex, the conformation of the protein could be fully open, two amino acid side-chains (his253 and tyr92) leaving the metal ion environment and allowing the low molecular mass ligand to coordinate to $V^{IV}O$ in a bidentate fashion (see Fig. 1B).

On the other hand, Garribba et al. identified several ternary species with $V^{IV}O$–apoTf through the exclusive use of frozen solution EPR [44,45]. Lac and cit were demonstrated among the low molecular mass serum constituents when the EPR spectra of the ternary complex differed form that of the binary (VOapoTf). In the cases of four other ligands in the serum (ox, phosphate, gly and his), the spectrum of the ternary system could be superimposed on that of the binary one. Oxalate [43] (and most probably phosphate) is able to behave as a synergistic anion only under certain conditions (e.g. in the absence of HCO_3^-), but is not able to compete with the hydrogen–carbonate anion under serum conditions. The situation is most probably the same with his and gly [44].

The same research group found that two drug candidate ligands, dhp and pic, also form ternary complexes with $V^{IV}O$–apoTf [45]. In contrast with our observations, besides 1:1:1 and 1:2:2 apoTf–VO–dhp complexes, they identified an apoTf–$VO(dhp)_2$ adduct on the bases of EPR parameters and DFT calculations, although they reported no further evidence (e.g. concentration dependence measurement) as to its composition. In the apoTf–$VO(dhp)_2$ adduct, they presume partial axial coordination of one of the bidentate ligands dhp (the *cis* form of $VO(dhp)_2$), while a histidine residue of the protein occupies the fourth equa-

Table 1
Conditional stability constants of V^{IV}–apoTf complexes at pH = 7.4.

$\log \beta_1$	$\log \beta_2$	Method	Detection	Conditions	Refs.
13.2 ± 1.6	–	Calculation, LFER	–	–	[25,31]
13.4 ± 0.2	11.9 ± 0.5	Ligand competition (NTA)	UV–vis	pH = 7.5 HEPES 0.1 M 25 mM $NaHCO_3$	[41][a]
13.4 ± 0.2	11.8 ± 0.4	Ligand competition (dhp, mal)	CD, RT EPR	pH = 7.5 HEPES 0.1 M 25 mM $NaHCO_3$	[12]
13.0 ± 0.5	12.5 ± 0.4	Ligand competition (acac)	LN EPR	pH = 7.4 HEPES 0.1 M 25 mM $NaHCO_3$	[13]

[a] In the same article higher values also have been published (in the range 14.0–15.0), but it was due to the ternary complex formation which was not considered at that time (see text).

torial position. The coordination mode is the same as in the *cis*-[VO(dhp)$_2$(MeIm)] (MeIm ≡ 1-methylimidazole) and the EPR parameters of their spectra also agree. However, in the ternary system of apoTf with picolinate no apoTf–VO(pic)$_2$ but only the species with 1:1:1 and 1:2:2 stoichiometries have been found (as in our case with dhp and mal). Furthermore, no ternary complexes have been identified with the third investigated ligand, acac. The ligand pic is believed to behave as a synergistic anion.

The determined stability constants and the EPR spectroscopic parameters of all the reported ternary complexes are listed in Table 2. Our observations concerning the dhp–V^{IV}O–apoTf ternary system have been criticized [45] in that, if the proposed coordination mode is correct, then all the ligands could form ternary complexes, and whereas acac, for instance does not. However, a similar problem arises with the apoTf–VO(dhp)$_2$ adduct in Ref. [45], which is formed only with dhp, and not with pic, although the existence of complexes HSA–VO(L)$_2$ (L = dhp, pic) with the same coordination mode was reported in the same article.

A comparison of the stepwise stability constants of the four reported 1:1:1 complexes V^{IV}O–apoTf–L (L: dhp, mal, pic and lac) with the conditional stability constants of the species VOL under the same conditions (Table 3) [23,27,46,47], permit the conclusion that only the value for dhp differ significantly from the others. However, the value is only 1.5 orders of magnitude higher, and it is difficult to say that the coordination modes must be completely different. It is possible that pic and lac form similar complexes to those of dhp, and do not behave only as synergistic anions as suggested by Garribba et al. [45]. Support for this hypothesis is that lac is not able to behave as a synergistic anion for both lobes of apoTf in the absence of HCO_3^- [43].

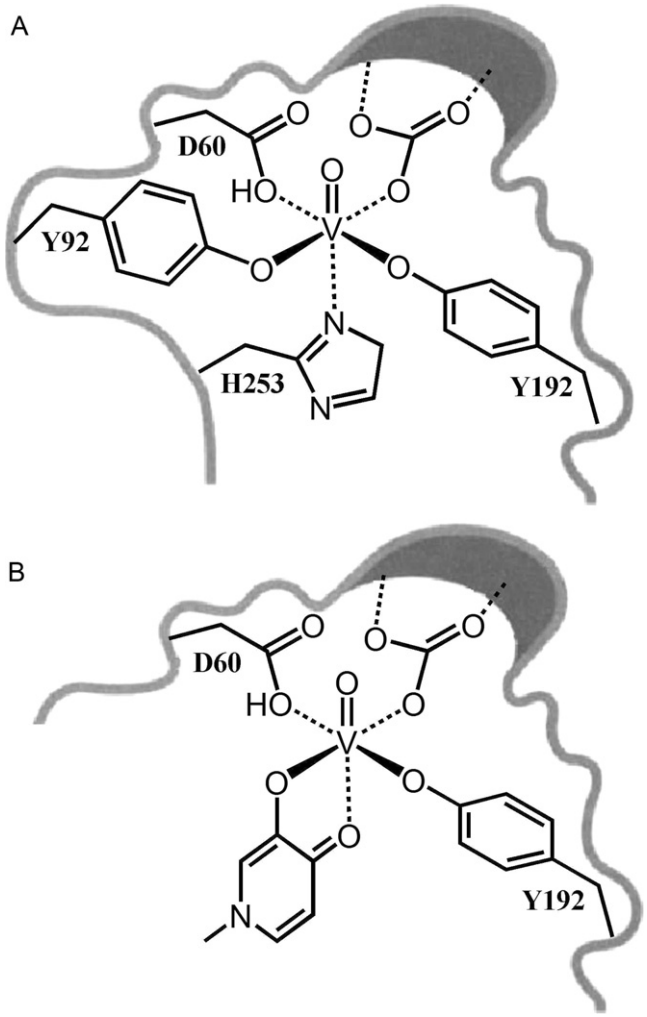

Fig. 1. Suggested binding modes for the V^{IV}OapoTf (A) binary and for the V^{IV}OapoTf(dhp) (B) ternary species.

8. V^{IV}O interaction with human serum albumin (HSA)

Unfortunately, the first study of serum albumin–V^{IV}O complexes (published by Chasteen and Francavilla) [48] involved the use of bovine serum albumin (BSA) at pH 5.0. Under those conditions, one "strong" and five weak binding sites were found, and the N-terminal primary Cu^{II} site was suggested for the "strong" binding position. Apart form small differences in linewidths, the spectra measured at RT and at LNT proved to be similar.

In studies with small oligopeptides, in the absence of a primary anchor donor group V^{IV}O ions (similarly to Cu^{II}) are not able to induce the deprotonation of the amide groups [49]. The N-terminal amino group is suitable for Cu^{II}, but not for V^{IV}O.

In another study (published by Sakurai et al.) of the V^{IV}O–BSA interaction [20], the LNT EPR spectra of V^{IV}O in rat blood, and in 4% BSA solution ($c_{protein} \sim 0.6$ mM; pH unknown) were compared at $c(V^{IV}O) = 400\ \mu M$; two species with different EPR spectroscopic parameters (see Table 4) were detected in both solutions. The same research group also studied the V^{IV}O–BSA interaction by means of CD spectroscopy [50] (0.1 M $NaClO_4$, pH 7.4 and 37 °C); a different binding site, containing the only free thiolate (Cys-34) group, was identified. BSA is able to bind one equivalent of Cu^{II} and 1.5 equiv. of V^{IV}O at the same time; no displacement reactions were observed, indicating that V^{IV}O binds to a different site than Cu^{II}. Iodoacetamide-modified BSA, which is in a partially thiolate-masked form, has limited V^{IV}O-binding ability; the intensity of the measured CD spectrum was reduced as compared with that of the original system. However, the CD intensity increased up to three equivalents of V^{IV}O *per* protein, which is in contradiction with the fact that there is only one free thiolate group (Cys-34) in serum albumins. Willsky et al. [39] mentioned that the complex HSA–V^{IV}O is very sensitive to specific solution conditions and aerobic oxidation.

The first EPR spectroscopic parameters on HSA at pH 7.4 were reported only in 2005 by Orvig et al. [40]. Garribba et al. detected a dinuclear $(VO)_2$HSA species by EPR at −150 °C [11]. The spin state of this species is 1, it is observed up to a V^{IV}O–HSA ratio of 1:1; the intensity of the spectrum is very low. The EPR parameters ($g_{||} = 1.981$, $80 \times 10^{-4}\ cm^{-1}$, $D = 631 \times 10^{-4}\ cm^{-1}$) suggest an

Table 2
Conditional stability constants and EPR parameters of the V^{IV}–apoTf–L terner complexes formed at pH = 7.4.

Ligand	Composition	log β	EPR				Refs.
	$VO_xapoTf_yL_z$		$g_\perp$	$A_\perp$[a]	$g_\parallel$	$A_\parallel$[a]	
mal	1:1:1[b]	17.7 ± 0.2	1.971(1)[d]	58.5(5)[d]	1.937(1)[d]	165.5(5)[d]	[12]
mal	2:1:1[b]	30.3 ± 0.3	–	–	–	–	[12]
mal	2:1:2[b]	34.8 ± 0.1	1.971(1)[d]	58.5(5)[d]	1.937(1)[d]	165.5(5)[d]	[12]
dhp	1:1:1[b]	21.3 ± 0.1	1.972(1)[d]	57.5(5)[d]	1.940(1)[d]	164.5(5)[d]	[12]
dhp	2:1:1[b]	30.05[c]	–	–	–	–	[12]
dhp	2:1:2[b]	40.3 ± 0.1	1.972(1)[d]	57.5(5)[d]	1.940(1)[d]	164.5(5)[d]	[12]
dhp	1:1:2	25.5 ± 0.6	–	–	1.947	163.3	[13,45]
pic	1:1:1	15.6 ± 0.8	–	–	1.944	167	[13,45]
pic	2:1:2	30.4 ± 0.8	–	–	–	–	[13,45]
lact	1:1:1	14.5 ± 0.8	–	–	1.939	167	[13,44]
lact	2:1:2	28.5 ± 0.8	–	–	–	–	[13]
cit	1:1:1	–	–	–	1.939	167	[13,44]

[a] The unit is $\times 10^{-4}$ cm^{-1}.
[b] pH = 7.5 HEPES 0.1 M, 25 mM $NaHCO_3$.
[c] Estimation, see Ref. [12].
[d] RT.

Table 3
Comparison of the conditional formation constant of the ternary ($V^{IV}O$)apoTf(L) complexes with the corresponding conditional stability constant (log K) of VO(L) at pH = 7.4.

Ligand	log K[a]	log $K^{cond}_{VO(L)}$	log K − log $K^{cond}_{VO(L)}$	Refs.
dhp	7.9	9.92	−2.0	[12,27]
mal	4.3	7.70	−3.4	[12,23]
pic	2.6	6.66	−4.1	[13,46]
lact	1.5	5.02	−3.5	[13,47]

[a] K: (VO)apoTf + L = (VO)apoTf(L).

Table 4
EPR parameters of the formed species in the $V^{IV}O$-serum albumin (SA) systems determined by different research groups.

SA	$xV^{IV}O$:SA	pH	EPR				Ref.
			$g_\perp$	$A_\perp$[a]	$g_\parallel$	$A_\parallel$[a]	
Bovine	1:1[b]	5.0	1.979	64.0	1.939	172.8	[48]
Bovine	≥2:1[c]	5.0	1.979	65.6	1.938	177.1	[48]
Bovine[d]	<1:1	?	–	–	1.940	173.5	[20]
Bovine[d]	<1:1	?	–	–	1.945	166.6	[20]
Human	1:1[b]	7.4	1.9628/1.9600[e]	61.70/55.48[e]	1.9265	166.5	[40]
Human	≥2:1[c]	7.4	1.9628/1.9600[e]	61.70/55.48[e]	1.9355	164.5	[40]
Human	≥1:1	∼5	–	–	1.946	171.2	[11]
Human	≥1:1	7.4	–	–	1.947	164.6	[11]
Human	≤1:1	7.4	–	–	1.981	80[f]	[11]

[a] The unit is $\times 10^{-4}$ cm^{-1}.
[b] So-called "strong" site.
[c] So-called "weak" site.
[d] Two species with different coordination mode were reported.
[e] Rhombic spectra: g_x/g_x or A_x/A_y. The simulated parameters were fixed for VO–HSA strong and weak.
[f] D value for the dinuclear $(VO)_2$HSA species is 631×10^{-4} cm^{-1}.

anti-coplanar arrangement of the two V=O bonds. At VO^{2+}/HSA > 1 another type of spectrum (spin 1/2) becomes predominant (the EPR parameters are given in Table 4). No other type of spectrum is observed up to a VO^{2+}:HSA ratio of 8:1; the EPR signal intensity reaches its maximum at a VO^{2+}:HSA ratio of ∼4:1. The authors used the $(V^{IV}O)_x{}^m$HSA abbreviation for these species. The conversion from a dimeric species to a monomeric one on increase of the metal ion excess appears contradictory; this was explained in terms of "binding mode of the metal ions changes for some reason" [11]. Our opinion is that the only way to describe this system with stability constants is the assumption of two types of independent $V^{IV}O$-sites: the first type (only one site) is able to bind two $V^{IV}O$ as a dimer, while the other type (one or more site) is able to bind one $V^{IV}O$ as a monomer. Actually, when Garribba et al. determined the stability constants of $(V^{IV}O)_2$HSA and ($V^{IV}O$)HSA complexes (see Table 5) via frozen solution EPR measurements [13] (where the stability constants of ($V^{IV}O$)HSA represent only a mean value for the association constants of $(VO)_x{}^m$HSA.), implicitly accepted the just mentioned independent site model.

The speciation of the $V^{IV}O$–HSA system calculated on data in Table 5 is depicted in Fig. 2, which clearly shows that, in the therapeutically important concentration range the ($V^{IV}O$)HSA is more important than $(V^{IV}O)_2$HSA, and under conditions of a metal ion excess the hydrolysis of $V^{IV}O$ does not allow the $(V^{IV}O)_x{}^m$HSA complex to reach its theoretical maximum.

Table 5
The conditional stability constants of the $V^{IV}O$–HSA complexes at pH = 7.4.

Complex	log β	Method	Ref.
$(V^{IV}O)_2$HSA	20.9 ± 1.0	EPR (−150 °C)	[11]
($V^{IV}O$)HSA	9.1 ± 1.0	EPR (−150 °C)	[11]

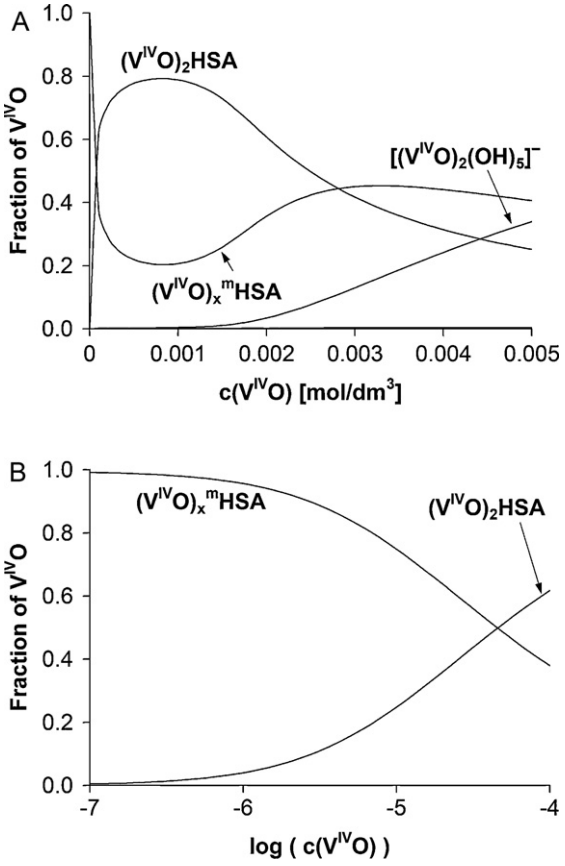

Fig. 2. Speciation curves of the complexes formed in the $V^{IV}O$–HSA system at pH 7.4 and 25.0 °C. *c*(HSA) = 0.63 mM) (A) $V^{IV}O$ excess, (B) serum concentration conditions (based on data reported in Ref. [13]).

9. $V^{IV}O$–HSA ternary complexes

Wilsky et al. suggested first ternary complex formation between HSA, $V^{IV}O$ and mal [39].

In the HSA–$V^{IV}O$–mal system, a signal, not present in the spectra of $V^{IV}O(mal)_2$ and HSA–$V^{IV}O$ was detected and characterized by Orvig et al. [40]. The spectrum of $V^{IV}O(mal)_2$ with an additional histidine-N coordination (using 1-MeIm) has the same EPR parameters as those of the observed ternary complex. Ternary complex formation with maltol has been confirmed and studied with other exogenous ligands in detail by Garribba et al. [13,44,45].

Similarly to the $V^{IV}O(mal)_2$ species [40], $V^{IV}O(pic)_2$ and $V^{IV}O(dhp)_2$ coordinate through a histidine side-chain, which binds in the equatorial position to the *cis* form (with the coordinated water *cis* to the oxido-O atom) of the bis $V^{IV}O$ complexes [45]. The existence of this type of species and coordination mode is proved by the fact that the addition of 1-MeIm to the bis complexes causes the same effect as in the case of HSA, and the EPR parameters of the detected spectra are very close to each other. In the case of the $VO(acac)_2$ complex, no ternary complex formation is observed with HSA. The stability constant of *cis*-$V^{IV}O(dhp)_2$HSA were found to be $\log K = 25.9 \pm 0.6$ [13].

The spin Hamiltonian parameters for several ternary species are depicted in Table 6.

10. Stability of V^V complexes – and interactions with low molecular mass serum constituents

Interactions of V^V with drug candidate ligands (mal, [51] and pic [52]) and low molecular mass serum constituents (lac [53], cit [54] and phosphate [55]) have mostly been studied by Pettersson et al.; his [56] and gly [57] also been mentioned [58] as potential V^V binders.

We have determined the stoichiometry and stability constants of the V^V complexes with hpno and mpno; and the stability of their bis complexes (VO_2L_2) under serum conditions, in comparison with three other drug candidate ligands (dhp, mal and pic). The stability sequence hpno > mal ~ dhp ≫ mpno > pic was reported [59]. However, our unpublished speciation data on the V^V–dhp system indicate that $[V^VO_2(dhp)_2]^-$ has a stability about one order of magnitude higher than the re-calculated data, which makes dhp the strongest V^V binder among these ligands.

The dipicolinate complex of V^V displays an antidiabetic effect [60], but the complex and a derivative [61] are not stable, and decompose at pH = 7.4. Studies have not yet been published on ternary complex formation in V^V–drug candidate ligands–serum constituent systems.

11. V^V interactions with apoTransferrin

ApoTf is able to bind two V^V, similarly to other metal ions, but in contrast with this the synergistic HCO_3^- anion is not involved in the coordination; V^V occupies the same site as Fe^{III} does. The interaction can readily be detected by differential UV titration [36,62], ^{51}V NMR [59,63,64], ultrafiltration [59,65] and calorimetry [42]; other special techniques, such as ^{14}N or ^{35}Cl NMR [19], have also been applied. The coordination takes place in the cationic form (VO_2^+): the stability constants of the apoTf–V^V interactions are 3–4 orders of magnitude higher than those with inorganic anions (phosphate, sulfate, hydrogencarbonate) [62]. Further evidence is that the molar absorbance difference caused by V^V-coordination to apoTf clearly indicates the deprotonation of the two tyrosine side-chains [36]. If we consider the coordination of the carboxylate and the histidine-N too, the two oxido groups complete the coordination sphere and the redundancy of the synergistic anion is understandable.

The two V^V ions, coordinated at the N- and C-terminal sites, can be distinguished through the use of ^{51}V NMR spectroscopy, which indicates a slight difference between the two binding sites [63]. However, there is not a highly preferred lobe: at a V^V:apoTf ratio of 1:1 both coordination pockets are partially occupied [59,63].

In contrast with these observations, competition between vanadate and HCO_3^- has been reported for the transferrin "anion" binding sites [62], and a similar suggestion has been published on the basis of the ^{13}C NMR data [64]. ^{51}V NMR measurements demonstrated that HCO_3^- cannot displace V^V from its apoTf complexes, and has no effect on the V^VapoTf + 2mal = apoTf + V^Vmal$_2$ ligand displacement reaction either. The observations in Ref. [62] can be explained only by assuming the formation of HCO_3^-–V^V adducts, similarly as for V^V-phosphate compounds.

The conditional (pH = 7.4) stability constants of the V^V–apoTf complexes (apoTf(V^V) and apoTf(V^V)$_2$) have been determined by ultrafiltration [59,65], calorimetry [42], difference UV-titration [62], and ^{51}V NMR [59] (ligand competition with mal and hpno). The constant for binding of the second V^V was also reported in Refs. [36,66].

Most of the $\log K_1$ data lie in the range 5.7–6.0 (see Table 7); the V^V binding abilities of the two sites appear to be equal as the difference between $\log K_1$ and $\log K_2$ is very close to the statistically determined value of $\log 4 = 0.6$. A stability constant $\log K_1$ higher by 1.5 orders of magnitude was determined because of the misconception of carbonate–vanadate competition. To date no ternary complex formation has been reported between V^V, apoTf and low molecular mass carrier ligands or serum components.

Table 6
EPR parameters of the ternary complexes formed in the $V^{IV}O$–carrier ligand–HSA and $V^{IV}O$–carrier ligand–MeIm systems.

Ligand	Complex	EPR				T (°C)	Ref.
		$g_\perp$	$A_\perp$[a]	$g_\parallel$	$A_\parallel$[a]		
dhp	$V^{IV}OL_2$(HSA)			1.947	162.1	−150	[45]
dhp	$V^{IV}OL_2$(MeIm)			1.947	163.0	−150	[45]
mal	$V^{IV}OL_2$(HSA)[b]	1.98/1.977	59.0/54.0	1.946	163.2	−143	[40]
mal	$V^{IV}OL_2$(MeIm)[b]	1.980/1.977	60.0/55.0	1.944	164.8	−143	[40]
pic	$V^{IV}OL_2$(HSA)			1.950	159.7	−150	[45]
pic	$V^{IV}OL_2$(MeIm)			1.943	158.8	−150	[45]

[a] The unit is $\times 10^{-4}$ cm^{-1}.
[b] Rhombic spectra: g_x/g_x or A_x/A_y.

Table 7
Conditional stability constants of the apoTf–V^V complexes at pH = 7.4.

$\log K_1$	$\log K_2$	Method	Ref.
5.67 ± 0.04	–[a]	Ultrafiltration	[65]
5.5 ± 0.1[b]		Calorimetry	[42]
6.02 ± 0.12	5.42 ± 0.12	Ultrafiltration	[59]
6.03 ± 0.10	5.46 ± 0.18	^{51}V NMR	[59]
7.45 ± 0.17	6.6 ± 0.3	Difference UV	[62]

[a] Bonding only one V^V to the apoTf was reported.
[b] The same value was published for the C- and N-lobe sites.

12. V^V interaction with human serum albumin

Unfortunately, V^V–HSA interactions are not as well known as in the case of the V^V–apoTf complexes. Similarly to $V^{IV}O$, V^V is not able to bind at the N-terminal copper binding site; only significantly weaker, non-specific interactions involving carboxylic functions for example, may be assumed.

No interaction between V^V and HSA is detected by ^{51}V NMR spectroscopy and fluorimetry [59]. Ultrafiltration results [65] indicate that V^V is bound to HSA only in very low concentrations relative to the total HSA (at most 0.3–0.4%); and the reported binding constants for V^V–HSA and V^V–apoTf are very similar; the two facts together suggest that the HSA applied contained some apoTf as impurity. Neglecting this "strong" interaction, a $\log K = 1.8 \pm 0.3$ can be calculated [59] from the published ultrafiltration data [65], which is significantly lower than $\log K$ of 3.0 given by Crans et al. [57].

13. $V^{IV}O$ speciation in serum

Chasteen et al. measured the frozen solution EPR spectra of $V^{IV}O$ in serum [19], but without publishing the EPR spectra and spectroscopic parameters of HSA–$V^{IV}O$ complex(es) at physiological pH (7.4). The metal ion concentration range applied was 25–100 μM. It was reported that at $c(V^{IV}O) = 25$ μM the metal ion is distributed between HSA and apoTF in a ratio of 2.3:1, which increases to 6.4:1 at $c(V^{IV}O) = 50$ μM. Addition of 50 μM apoTf reduces the ratio from 6.4:1 to 2.1:1. At $c(V^{IV}O) = 100$ μM in the serum, when the vanadium added as vanadate and reduced by the serum under a N_2 atmosphere, the 100% reduction to $V^{IV}O$ is proved by double integration of the spectrum signal, which means that no significant amount of EPR-silent species (e.g. V^{III}, V^V or dimer $V^{IV}O$) exist in the serum under these conditions. This was the first and only trial in which the chemical form of the $V^{IV}O$ in the serum was determined by use of an "*in situ*" spectroscopic technique. The authors also determined the relative $V^{IV}O$ binding strength of apoTf to HSA (K_{apoTf}/K_{HSA} ratio ~ 6) [19], but its value seems to be far from the reality, most probably due to the misinterpretation of their EPR spectra. Later results [11,12] agreed that the same value has to be at least three/four times order of magnitude higher. After determination of the stability constants of the $V^{IV}O$ complexes of many serum constituents by pH-metry and other methods, modeling calculations of the $V^{IV}O$ speciation become feasible. The speciation (see Fig. 3B) for $V^{IV}O(mal)_2$ clearly showed that $V^{IV}O$ concentrations <10 μM all of the $V^{IV}O$ is bound to exclusively to apoTf, and the same should hold for all the complexes with similar or lower conditional stability (e.g. $V^{IV}O(pic)_2$, $V^{IV}O(Me\text{-}pic)_2$, $V^{IV}O(mpno)_2$ and $V^{IV}O(acac)_2$). The ternary complexes detected in the $V^{IV}O$–apoTf–mal system are not stable enough to form under the same conditions in the serum.

In order to have at least 10% $V^{IV}OA_2$ complex at 10 μM $V^{IV}O$ in the serum, the conditional stability constant ($\log \beta$) of the complex should be higher than 17.15. This is valid only for the $V^{IV}O(dhp)_2$ complex where, $\log \beta_{cond} = 18.11$. ($\log \beta_{cond} = 15.99$ [28] for $V^{IV}O(hpno)_2$, 14.13 [23] for $V^{IV}O(mal)_2$, 13.51 [67] for

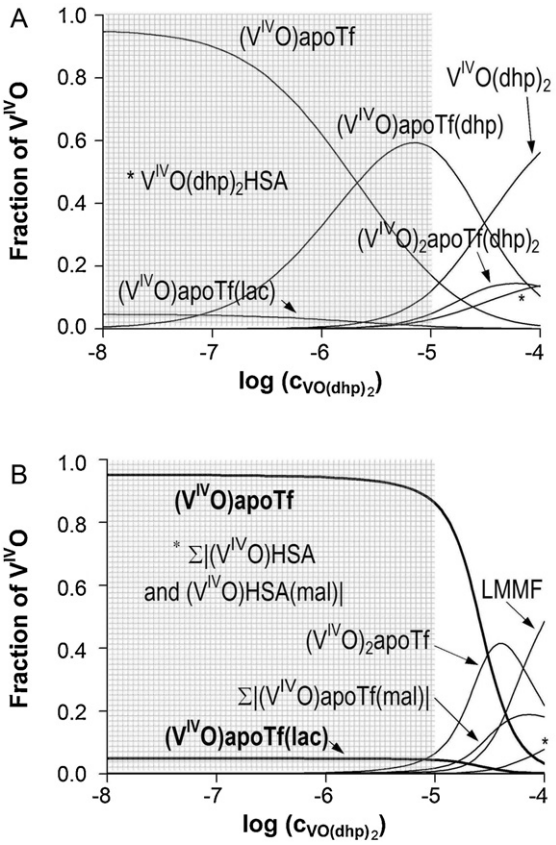

Fig. 3. Calculated speciation in the blood serum: (A) $V^{IV}O(dhp)_2$, (B) $V^{IV}O(mal)_2$, (pH = 7.4, $c(HCO_3^-) = 25.0$ mM, $T = 25$ °C). The textured areas represent the concentration range relevant to oral administration of V (≤10 μM) (based on data reported in Refs. [12,13]) (LMMF: low molecular mass fraction, it means all the species do not contain apoTf or HSA).

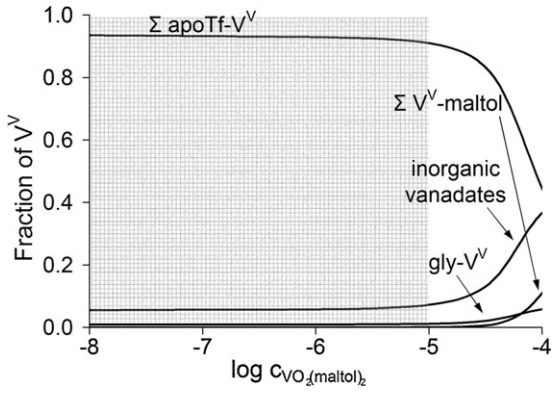

Fig. 4. Calculated speciation in blood serum for $[V^{V}O_2(mal)_2]$ (pH = 7.4, $c(HCO_3^-)$ = 25.0 mM, T = 25.0 °C). The textured areas represent the concentration range relevant to oral administration of V (≤10 μM) (based on data reported in Ref. [59]).

$V^{IV}O(acac)_2$, 13.0 [28] for $V^{IV}O(mpno)_2$, 12.06° [24] for $V^{IV}O(pic)_2$, and 9.26 [24] for $V^{IV}O(Mepic)_2$). However at 10 μM $V^{IV}O$, due to ternary complex formation, mostly the complex (VO)apoTf(dhp) (and some of the original complex) containing the carrier ligand show up in the speciation model (see Fig. 3A). The interactions between the HSA and drug candidate bis complexes through imidazole-N atoms can increase their conditional stability by only ~0.1–0.5 log unit, which is negligible, as the interactions themselves.

Garribba et al. utilized frozen solution EPR measurements to redetermined the stability in the $V^{IV}O$–apoTf and $V^{IV}O$–HSA systems and found new binary and ternary species. There are some differences between our and their model, but both give basically the same picture; $V^{IV}O$ is transported solely by apoTf, if the concentration of this antidiabetic compounds is <10 μM (with the exception of dhp). Of course if $c(V^{IV}O)$ is > than some tens of μM, i.e. when it is in excess or in comparable concentration with apoTf, than apoTf is saturated with and is not able to bind all of the $V^{IV}O$ present, the organic carriers and HSA can be involved in its transport [14].

14. V^{V} speciation in serum

The first studies on V^{V} speciation in serum were published by Pettersson et al. [58]. Their model gave practically the right result: V^{V} complexes (except with apoTf) can be observed only in the presence of an enormous amount (5000–20,000-fold excess) of carrier ligand. Redetermination of the stability of the V^{V}–apoTf complexes [59] led to only minor changes in the speciation (see Fig. 4); at <10 μM V^{V}, only the complex V^{V}–apoTf is formed, though the content of free V^{V} (~5%) is not completely negligible.

15. Conclusions

The use of modeling calculations to determine the speciation of vanadium in serum has both advantages and disadvantages. It can treat systems with fast dynamic processes, and furnish information on those concentration or ratio ranges where the spectroscopic tools are no longer useful. It is not a perfect method because it is impossible to carry out an independent study of all the interactions which can occur in serum. Most of the stability constants were determined under conditions (e.g. 25 °C) different from those in the living blood serum. A higher protein concentration for example, can increase the number of protein–protein interactions, and in the case of metal to ligand ratios, such as 1:1000, different species may exist [68] than under normally applied conditions. Moreover, if an adduct consists of 4–5 or more components (e.g. apoTf, Fe^{III}, $V^{IV}O$, dhp and HCO_3^- can form one species), determination of the stability constant is almost impossible as the number of subsystems to be known rises exponentially. It is also true that calculations can afford information only about the thermodynamic equilibria, which are not reached in the blood serum. Notwithstanding these negative aspects, modeling calculations extended with result obtained through use of separation techniques (e.g. ultrafiltration, chromatography and capillary electrophoresis) can yield important information about the plausible biotransformations of drug candidate compounds in the blood serum.

Under therapeutically relevant conditions ($c_V \leq 10$ μM), vanadium in oxidation state IV and V is bound to apoTf (or Tf in the blood) solely at the Fe^{III} free binding sites; all the original carrier ligands are displaced. The only exception is the ligand dhp, which forms ternary complexes with $V^{IV}O$ and apoTf sufficiently stable to play a role in the speciation in the serum. Thus $V^{IV}O(dhp)_2$ should have unique properties among the antidiabetic $V^{IV}O$ compounds; although its activity in cell tests [69,70] is controversial. This can be explained in two different ways: in the ternary complex formed, the apoTf is in an open form and not able to bind to the receptor and thus the metal ion is not transported into the cell, or the very stable compounds are simply not efficient in antidiabetic therapy [1].

The results of modeling calculations, together with the results of the separation measurements and complemented with the pharmacokinetic observation that BEOV dissociates soon after oral gavage administration [71], lead to the conclusion that the vanadium ion part of these insulin-enhancing drug compounds itself is the active metabolite responsible for the antidiabetic effects.

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