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ORIGINAL PAPER

Study of tungstate–protein interaction in human serum by LC–ICP-MS and MALDI-TOF

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Abstract Oral administration of sodium tungstate is an effective treatment for type 1 and 2 diabetes in animal models; it does not incur significant side effects, and it may constitute an alternative to insulin. However, the mechanism by which tungstate exerts its observed metabolic effects in vivo is still not completely understood. In this work, serum-containing proteins which bind tungstate have been characterized. Size exclusion chromatography (SEC) coupled to inductively coupled plasma mass spectrometry (ICP-MS) with a Phenomenex Bio-Sep-S 2000 column and 20 mM HEPES and 150 mM NaCl at pH 7.4 as the mobile phase was chosen as the most appropriate methodology to screen for tungsten–protein complexes. When human serum was incubated with tungstate, three analytical peaks were observed, one related to tungstate–albumin binding, one to free tungstate, and one to an unknown protein binding (MW higher than 300 kDa). Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometric analysis of the tungsten-containing fractions collected from SEC–ICP-MS chromatograms, after desalting and preconcentration processes, confirmed the association of tungstate with albumin and the other unknown protein.

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Introduction

Several inorganic substitutes for insulin, such as vanadate, selenate and lithium salts, have been described [1]. In several animal models with type 1 and 2 diabetes, sodium tungstate reduces and, in most cases, normalizes glycemia when administered orally [2]. In addition, several reports have also shown antidiabetic actions for the tungsten derivates paratungstate and pertungstate [3, 4]. In contrast to other transition metal derivatives, sodium tungstate has a low toxicity profile in both short- and long-term treatments [5, 6]. Long-term tungstate treatment reduces body weight gain and the occurrence of various complications of diabetes in rats, such as morphological changes in kidney and ocular lens, and also reduces mortality but does not cause any undesirable side effects [7]. Despite numerous studies, the mechanism by which tungstate performs its observed metabolic effects in vivo is still not completely understood. Thus, more research is still required to clarify the biochemical forms and physiological functions of this element in humans.

Elemental speciation studies using hyphenated techniques such as liquid chromatography coupled to inductively coupled plasma mass spectrometry (LC–ICP-MS) can be a valuable tool for obtaining information about the biomolecules that may be involved in the biochemistry of tungsten and its transport and storage in body fluids and tissues [8, 9].

Several separation techniques, such as gel electrophoresis [10], size-exclusion (SEC) [11] or ion exchange chromatography [12–14] have been proposed for the sepa-

ration and determination of metal biocompounds. SEC with on-line detection by ICP-MS appears to be the primary technique that allows metals bound to macromolecular ligands in an unknown sample to be detected [15]. It is known that SEC has the ability to separate biomolecules into ranges of different molecular weights, and ion exchange in the anion-exchange mode also offers an interesting alternative for the separation of metal biocompounds [12, 13]. The use of anion exchange fast protein liquid chromatography (AE-FPLC) coupled with ICP-MS has provided satisfactory results when studying protein binding to metals such as aluminum, iron and vanadium [16, 17].

In the present study, we have chosen to use AE-FPLC and SEC coupled to ICP-MS detection in order to study the association of tungstate with proteins in human serum. Additionally, matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF-MS) will be used to characterize the protein moieties associated with tungsten fractions present in human serum.

Experimental

Instrumentation

For tungsten determination, a Quadupole ICP-MS Thermo X-Series (Thermo Electron, Windsford, UK) equipped with a Meinhard nebulizer, a Fassel torch and an Impact Bead Quartz spray chamber cooled by a Peltier system was employed. The ICP-MS operating conditions were: forward power 1250 W, plasma gas 15 L min^{-1}, auxiliary gas 0.73 L min^{-1}, nebulizer gas 0.85 L min^{-1}, channels per AMU 10, and integration time 0.6 ms. For total tungsten determination, sample introduction was performed in continuous mode and single-ion monitoring of *m/z* 184 (W), 186 (W) and 191 (Ir) was used to collect data. 100 μg L^{-1} of Ir was used as the internal standard.

For the speciation studies, which involved chromatographic separations, a high-pressure quaternary gradient pump (Jasco PU-2089, Tokyo, Japan) equipped with a 20 μL sample injection valve (Rheodine, Rohnert Park, CA, USA) was used as a sample delivery system. Separation of the proteins associated with tungstate was performed by SEC using a Phenomenex Bio-Sep-S 2000 (300 mm ×7.8 mm analytical gel filtration column, Pharmacia Biotech, Uppsala, Sweden) with a separation range of 1–300 kDa, and with a strong AE-FPLC Mono-Q H/R 5/5 column (50 mm×5 mm i.d., Pharmacia,, both running at a flow rate of 1 mL min^{-1}. The ICP-MS transient signal mode was set to chromatographic acquisition, monitoring *m/z* 184 (W), 66 (Zn) and 65 (Cu).

UV measurements were performed with a SpectroMonitor 5000 photodiode array detector (LDC Analytical Thermo Separation Products, Uxbridge, UK).

Molecular mass analyses of different proteins associated with tungsten were performed by MALDI-TOF-MS on a Voyager™ Biospectrometry™ Workstation (Applied Biosystems, Langen, Germany). The operating parameters employed were: positive mode 25 kV, 2600 A laser intensity, 100 laser shots.

A 5404R centrifuge (Eppendorf, Hamburg, Germany) was used.

Reagents and materials

All chemicals were of analytical grade quality at the very least. Human protein standards [albumin (HSA), apo-transferrin (apo-Tf), immunoglobulin G (IgG) and α_2-macroglobulin] were obtained from Sigma (St. Louis, MO, USA). Stock solutions of tungstate (1000 mg L^{-1} as tungsten in 5% nitric acid/trace hydrofluoric acid; Merck, Darmstadt, Germany) and Ir (1000 mg L^{-1} in 8% HCl, Merck, Darmstadt, Germany) were diluted with 5% HNO_3 to prepare standard solutions. The different mobile phases were prepared with tris(hydroxymethyl)-aminomethane (Fluka Chemie, Buchs, Switzerland), ammonium acetate (Merck), acetic acid (Merck), hydrochloric acid (Merck), NaCl and KCl (Panreac Quimica SA, Castellar del Vallès, Barcelona, Spain), HEPES [4-(2-hydroxyethyl)piperazine-1-(2-ethanesulfonic acid)] (Sigma-Aldrich, Bornem, Belgium) and NaOH (Merck). Sinapinic acid (Fluka, Steinheim, Germany), trifluoroacetic acid (Aldrich, Steinheim, Germany) and acetonitrile (Merck) were used for MALDI-TOF analysis. All solutions were prepared with deionized water (Milli-Q Ultrapure water systems, Millipore, MA, USA). High-purity nitric acid was obtained by distillation of the analytical grade reagents (Merck).

Fresh human serum was obtained from a healthy volunteer, and was stored at –18 °C and defrosted immediately prior to use.

Procedure used for in vitro studies

Independent solutions of 5 g L^{-1} HSA, 0.5 g L^{-1} apo-Tf, and 1 g L^{-1} IgG were incubated with 5 μg L^{-1} WO_4^{2-} solution (as tungsten) (previously neutralized at pH 7.0) at 37 °C for 1, 24, 48 and 96 h in a saline solution containing 0.9% NaCl and 10 mM HEPES buffer (pH 7.4) in order to mimic physiological conditions. Aliquots of the incubated solutions were further analyzed by LC–ICP-MS.

Freshly sampled human blood was separated into serum and blood cells by centrifugation at 6000×*g* for 20 min, the supernatant being transferred to a second vial and centrifuged a second time following the same procedure. It should be noted that no particular precaution was taken to prevent contamination of this serum from external sources, such as stainless steel needles. Aliquots of this serum were

incubated with 5 μg L^{-1} WO_4^{2-} at 37 °C for 1, 24, 48 and 96 h. All serum samples were diluted (1+1) with mobile phase before injection into the chromatographic system. All of the samples were filtered through a 0.22-μm Millex-GV_{13} filter (Millipore) before injection onto the column of the LC–ICP-MS system for tungsten detection. In parallel, LC–UV was used for protein monitoring at 280 nm.

Two fractions of the eluted human serum sample with high tungsten contents were collected from the SEC column at 6.6 min and 5.6 min (maximum peaks of HAS–W and unknown protein–W, respectively; 1 mL each) in polypropylene containers. This procedure was repeated twice to give a final volume of 2 mL of each fraction. These fractions were desalted and preconcentrated in a centrifugal filter device (using Centricon YM-30 cut-off filters from Millipore) by centrifugation at 5000×*g* and 20 °C for 1 h. The retentate was made up to 200 μL with ultrapure water. These aliquots were mixed with a saturated sinapinic acid solution in 0.1% (v/v) trifluoroacetic acid:acetonitrile for molecular mass protein identification by MALDI-TOF.

Results and discussion

LC–ICP-MS method development

Most biological systems are sensitive to small changes in pH, so the pH values of the mobile phases and the solutions in the in vitro incubation experiments should be carefully controlled during the experiment using a buffer solution. However, the potential interaction of metal–protein complexes with the stationary and mobile phases of the chromatographic system could result in the poor recovery of the target analyte [14, 17]. It is important to choose mild separation chromatographic methods to avoid artefacts in the resulting chromatograms. As no study related to the interaction of tungstate with serum proteins has been reported in the literature as yet, the development of a suitable chromatographic method for tungstate–protein separation and identification is mandatory. Thus, the suitabilities of two chromatographic columns based on different separation mechanisms in combination with several mobile phases were evaluated. The combination of either a Mono-Q H/R 5/5 (strong anion exchange FPLC column) or a Phenomenex Bio-Sep-S 2000 (1–300 KDa, SEC gel filtration column) with different mobile phases was evaluated.

Most serum proteins are glycoproteins, 52% of which are albumin [18]. Other minority proteins are transferrin, immunoglobulin G and macroglobulins, which have been reported to bind and transport metal ions in human serum.

As HSA and apo-Tf have been reported to interact with iron, aluminum and vanadium [8, 16, 19] (among other species), 5 g L^{-1} HSA was incubated with 10 μg L^{-1} tungstate for 24 h under physiological conditions (corresponding to the different chromatographic mobile phases tested), and the incubated sample was injected into the LC–ICP-MS system for tungstate–HAS detection. In parallel, a 10 μg L^{-1} tungstate solution was also injected onto each column to control the elution time of both free tungsten and those associated with HSA, and to evaluate the recovery of tungsten from the column. The results obtained, shown in Table 1, shown that tungsten (whether free or associated with protein) is strongly retained in the Mono-Q column for the different mobile phases tested. It is known that tungstate forms isopolytungstates with a high anionic charge at neutral pH. The presence of this highly charged species indicates that this polyanion is strongly retained on the anionic exchange column. However, the situation is significantly different for that reported for vanadium, which also forms polyanions [14].

In contrast, when the Phenomenex Bio-Sep-S 2000 SEC column was used, quantitative recovery was achieved only when HEPES buffer was used as the mobile phase in isocratic mode. Similar results for vanadium associated with proteins have been reported by other authors, which stated that HEPES, unlike to other buffers, does not interact with vanadium in solution [14]. Furthermore, an additional advantage of using a SEC column with HEPES as the

Table 1 Chromatographic conditions tested for the separation of tungstate complexes with human serum proteins

Column	Mobile phase	W–protein recovery	Inorganic W recovery
Mono Q HR 5/5 (AE-FPLC)	A: 50 mM tris-HAc, pH 7.4 B: 50 mM tris-HAc/250 mM NH_4Ac, pH 7.4	No	No
	A: 50 mM tris-HCl, pH 7.4 B: 50 mM tris-HCl/250 mM NH_4Ac, pH 7.4	No	Yes
	A: 20 mM HEPES, pH 7.4 B: 20 mM HEPES/150 mM NaCl, pH 7.4	No	Yes
Phenomenex Bio-Sep-S 2000 (SEC)	25 mM Tris-HCl/50 mM KCl, pH 6.8	<30%	Quantitative
	20 mM HEPES/150 mM NaCl, pH 7.4	Quantitative	Quantitative

Gradient elution conditions: A starting buffer, B terminal buffer

mobile phase is that the chromatogram is obtained more rapidly (14 min) than when obtained using the Mono-Q column with other mobile phases (30 min). This fact is especially relevant to mobile phases with a high saline content because it provides a drastic decrease in signal drift when ICP-MS is used as the detector. Thus the SEC column with 20 mM HEPES and 50 mM NaCl at pH 7.4 as mobile phase was selected for further study. We checked that this chromatographic system could work contamination-free, despite of its stainless steel components.

Tungstate–protein binding kinetics

The interactions of tungstate with human serum proteins were evaluated by incubating individual standards of 5 g L^{-1} HSA, 0.5 g L^{-1} apo-Tf and 1 g L^{-1} IgG with this metallic species (5 μg L^{-1} of tungsten). The different mixtures (tungstate–protein) were incubated at 37 °C for 2, 24, 48 and 96 h in saline solution (5 mM HEPES and 150 mM NaCl at pH 7.4).

The ICP-MS chromatographic profiles of W-complexes with different proteins after 2 h of incubation are shown in Fig. 1. In addition, the proteins were also spectrophotometrically detected at 280 nm. Good chromatographic resolution between free tungstate and both W-proteins was obtained, with the inorganic tungstate being the last species eluted in the chromatogram (9.5 min). All tungsten species eluted in 11 min with relatively symmetrical peaks and show no significant tailing. However, tungstate bound to HSA and apo-Tf coelute at a retention time of 6.5 min.

As can be observed in Fig. 1 and Table 2, tungstate forms complexes with HSA and apo-Tf, but not with IgG. About 40% and 10% of the tungstate interacts with HSA and apo-Tf, respectively, for an incubation time of 2 h. Prolonging the incubation time did not increase the complexation, which indicates that a fast kinetic process occurs for this kind of binding, which is far from being quantitative. It has been already reported that the presence of hydrogen carbonate anion seems to be required for adequate binding of V, Fe and Al ions to apo-Tf [19]. Thus, parallel incubation experiments with HSA or apo-Tf and tungstate in 0.025 M sodium hydrogen carbonate were carried out; however, no W–protein complex was observed in this medium.

Since the serum concentration of HSA is considerably greater than that of Tf, most tungstate in serum should be bound to HSA instead of Tf.

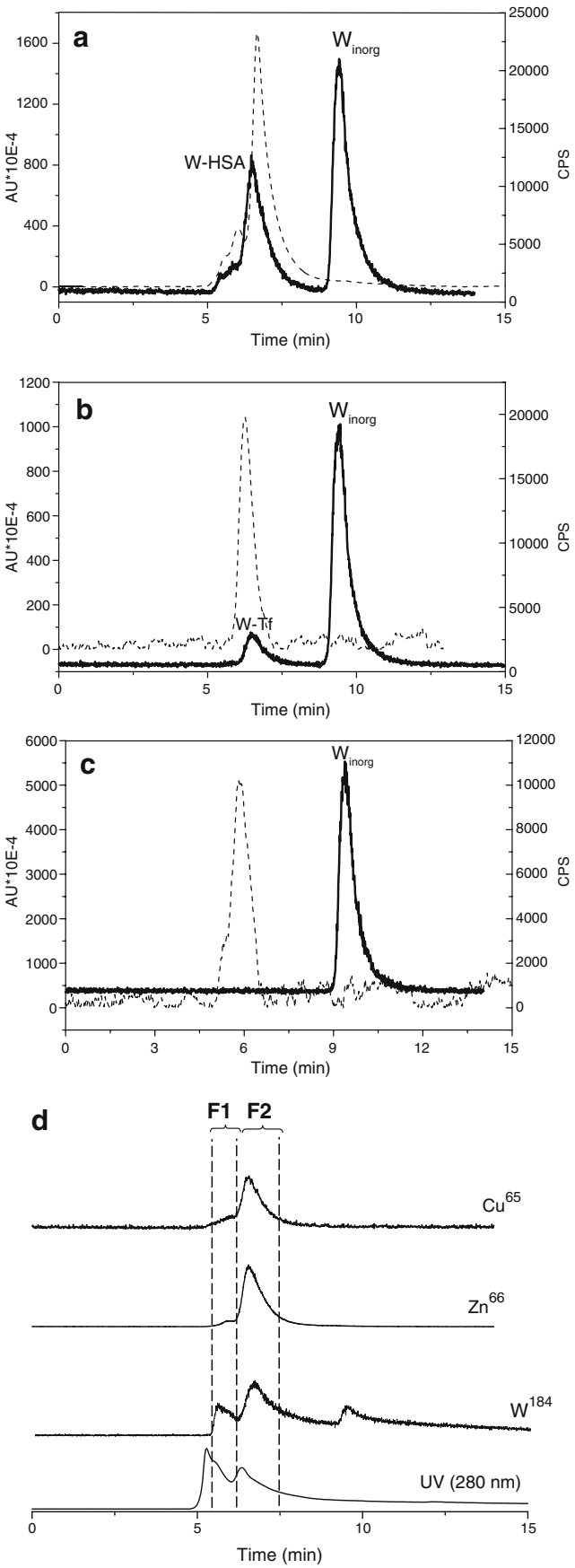

Fig. 1 SEC–ICP-MS (*dashed line*) and UV–HPLC (*dotted line*) chromatograms of standard solutions of human serum proteins incubated with 5 μg L^{-1} tungsten and : (**a**) 5 mg L^{-1} HSA; (**b**) 0.5 mg L^{-1} apo-Tf; (**c**) 1 mg L^{-1} IgG; and (**d**) human serum

Table 2 Binding efficiency of 5 μg L^{-1} tungstate (W) to different serum proteins after 2 h of incubation

Values given are the extent of tungstate–protein binding (%) after tungstate is spiked into human serum.

$[WO_4^{2-}]$, μgL^{-1}	Matrix	Unknown–W	Ig–W	Apo–W	HSA–W	$W_{inorganic}$
5	HAS	–	–	–	40	60
5	Apo-tf	–	–	11	–	89
5	IgG	–	–	–	–	100
5	Serum	20	–	–	45	35
10	Serum	22	–	–	47	31
50	Serum	19	–	–	42	39

Binding of tungstate to human serum proteins

The total content of tungsten in human serum samples from a healthy subject was lower than the detection limit of the ICP-MS (5 ng L^{-1}). The serum samples were incubated at 37 °C with 5 μg L^{-1} of tungstate for 2, 24, 48 and 96 h. Three analytical peaks corresponding to the presence of different species (free and bound) of tungsten were detected (Fig. 1d) after 2 h of incubation time. The chromatographic peaks at 6.6 min (F2) and 9.5 min match those previously obtained for tungstate complexed to HSA and/or apo-Tf and free tungstate, respectively. However, the analytical peak eluted at 5.6 min does not match any of the previously characterized tungsten compounds, and corresponds to the exclusion volume of the column. Therefore the MW of this W–protein should be higher than 300 kDa. It is important to note that, as seen for the protein standards, incubating for of >2 h did not result in any significant improvement in the extent of protein binding.

Considering that the interaction of α_2-macroglobulin with zinc and molybdenum in serum samples has already been reported [20], and that in some proteins molybdenum can be replaced by tungsten [20], the possibility that the unknown tungsten species detected at 5.6 min (F1) could correspond to a tungstate complex with α_2-macroglobulin was evaluated. Thus, a solution of 1.5 g L^{-1} α_2-macroglobulin was incubated with 5 μg L^{-1} of tungstate at 37 °C for 2 and 24 h, but no interaction was observed.

In order to check the effect of the tungstate concentration on the protein binding efficiency, three different concentrations of tungstate (5, 10 and 50 μg L^{-1}) were spiked into the human serum which was then incubated for 2 h. The results obtained (Table 2) show that the percentage of free inorganic tungsten remains almost constant in all cases, independent of the initial tungsten concentration used.

In order to test the reproducibility of the developed method, three human serum samples were incubated with 5 μg L^{-1} of tungstate for 2 and 24 h. The analysis of each incubated serum was performed in duplicate and the RSD for each identified tungsten species was in the range 2–7%. A comparison of the means using the Snedecor test did not indicate that there was any significant difference between both incubation series for α (0.05, n=3).

Taking advantage of the multielemental capabilities of ICP-MS, the chromatographic profiles of other essential elements (such as zinc and copper) associated with human

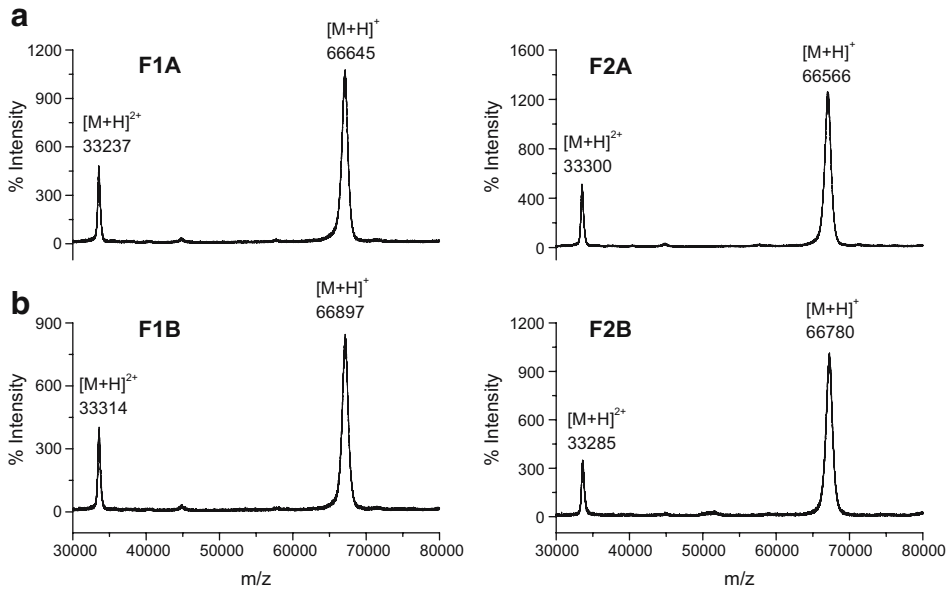

Fig. 2 MALDI-TOF spectrums observed for fractions F1 and F2 collected from SEC–ICP-MS, corresponding to (**a**) a human serum sample F1A, F2A and (**b**) a human serum sample incubated with 10 μg L^{-1} tungstate F1B, F2B

serum proteins were monitored. The fact that similar chromatographic profiles were obtained for zinc, copper and tungsten (Fig. 1) could lead us to assume that tungstate is associated with the same proteins as these other two essential elements. The main difference found was the presence of both free and bound tungstate to proteins, which was not observed for the other essential elements.

Identification of proteins bound to tungstate by MALDI-TOF

Unaltered protein analysis is one of the capabilities of the MALDI-TOF technique. Since most proteins obtain charges of +1 and/or +2 in this source, the resulting mass spectra are much easier to interpret than those obtained using an electrospray ionization (ESI)-MS. Furthermore, the sample treatment required for MALDI-TOF is simpler than that required for ESI-MS, where samples need to be free of significant salt content. Thus, the molecular weights of proteins can easily be calculated directly from the MALDI-TOF mass spectra. In order to check whether the two main peaks observed in the SEC–ICP-MS chromatograms could be related to differences in molecular weight, the two fractions (F1 and F2 in Fig. 1) were collected, desalted, preconcentrated ten times using Centricon YM-30 centrifugal filters, and finally analyzed by MALDI-TOF.

Mass values were calculated as the means and standard deviations of nine different sample spots. All mass spectra were recorded and averaged over 100 laser shots. Figure 2 shows the MALDI-TOF mass spectra obtained for fractions F1 and F2, corresponding to an unspiked human serum sample (A) and human serum spiked with 10 $\mu g\ L^{-1}$ tungstate (B). External calibration of the instrument was achieved by placing several aliquots of a standard solution of HSA (MW 66,554) in alternative holes of the MALDI plate.

To confirm that fraction F2 contained tungstate associated with HSA, the spectra of sera with and without tungstate were compared. However, it should be noted that albumin gives a wide peak in MALDI-TOF, which limits the accuracy of the molecular mass obtained. The molecular ion $(M+H)^+$ for the unspiked serum (F2A) was observed at *m/z* 66,566±25 Da (Fig. 2, F2A) very close to the theoretical value for HSA (66,554 Da). No peaks were obtained for apo-Tf (76,000 Da) in this fraction. The mass difference between the peaks in the spectra for fraction F2 serum samples with (F2B) and without (F2A) tungstate incubation was 214 Da, which can be attributed to tungstate binding (248 Da). The fact that the protein–tungstate binding was detected by MALDI-TOF contrasts with the data reported for vanadium–transferrin binding [19], where the integrity of the vanadium–protein binding was not maintained during the MALDI process. In our case we can assume that HSA is one of the proteins responsible for binding to tungstate.

Parallel experiments with fraction F1 gave a molecular ion $(M+H)^+$ for the unspiked serum (Fig. 2, F1A) at *m/z* 66,645±62 Da, very close to the theoretical of HSA. These results can be attributed to either the contamination of this fraction with HSA (it should be noted that the F1 and F2 chromatographic peaks are not completely resolved), or to the presence of a protein with a molecular weight similar to that of HSA. Furthermore, a molecular mass increase of 252 Da was observed when tungstate was present in the incubation (F1B), which again confirms that the tungstate binds to this protein.

Conclusions

An analytical screening method which allows the binding of tungstate to serum proteins to be identified has been developed for the first time. The chromatographic method—which is based on the use of SEC–ICP-MS coupling—lasts 15 min, which is much shorter than the time required for other antidiabetic target compounds.

MALDI-TOF analysis of SEC tungsten-containing fractions confirmed that albumin complexes tungstate. The presence of other unknown W–protein peaks shows that more research in this area is required using an alternative analytical methodology.

An important feature of the binding of the tungstate to serum proteins is the fact that, in all cases, and independent of the tungstate concentration used, the presence of free tungstate was detected.

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