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Rapid and sensitive LC-MS approach to quantify non-radioactive transition metal impurities in metal radionuclides†

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A rapid and sensitive LC-MS approach for the quantification of non-radioactive metal contaminants present in metal radionuclide formulations was developed. Traditional $^{12}C/^{1}H$ and heavy stable isotope $^{13}C/^{2}H$-labeled chelator–amino acid conjugates are used as chelating agents to quantify contaminating transition metals, allowing for determination of effective specific activity of radio-metals.

Metal-based radiopharmaceuticals have been widely used for radiotherapy and diagnostic imaging, and in recent decades, their development has been a rapidly growing area for both clinical and pre-clinical studies.[1–3] Radio-metals typically contain significant amounts of cold metal impurities that impede effective radiolabelling at high specific activities.[4,5] Achieving high specific activity (the amount of radioactivity per unit of mass) is extremely important for imaging agents that target proteins, such as tumour receptors, present in very low (nM or less) concentrations *in vivo*.[6,7] The effective specific activity (ESA) of a radio-metal is typically obtained by radiolabelling titrations with DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) and/or TETA (1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid).[8,9] However, the titration method will not provide information on the identity of the contaminating metals or the relative amounts present, and additionally suffers from inconsistent results that are dependent on the chelator and/or the labelling conditions used. Although ion chromatography (IC) has also been used to analyse metal impurities,[10] it is limited by low sensitivity, and requires high concentrations of radio-metals that can be hazardous from a radiation safety standpoint. To overcome these problems, a rapid and sensitive LC-MS approach using traditional $^{12}C/^{1}H$ and stable isotope $^{13}C/^{2}H$-labeled agents was developed. Through this methodology, not only quantification of non-radioactive metal contaminants in metal radionuclides can be achieved, but also the ESA of radio-metals for a particular chelator can be determined. Furthermore, this LC-MS method allows for optimization of the radiolabelling conditions to achieve high specific activity by using conditions favouring the chelation of a particular radio-metal.

Stable isotope labelling agents, such as ICAT, CILAT, iTRAQ, and SILAC, have been developed and widely used for quantitative proteomics with high precision.[11–18] The use of stable isotope-labelled tags involves the addition of a chemically identical form of the analyte(s) containing a stable heavy isotope (*e.g.*, ^{2}H, ^{13}C, ^{15}N, *etc.*) to a solution containing an internal standard. Due to the ionization variability for different compounds, the best internal standard for quantification is typically the same compound labelled with a stable isotope(s). The internal standard and analyte differ only by the incorporation of either the heavy stable isotopes, or the native stable isotopes. In theory, all compounds in the combined sample exist as analyte pairs of identical sequence but differing masses. The compounds have the same properties and behave with identical characteristics under any isolation or separation step. Thus, the ratio between intensities of the pair of compounds provides an accurate measurement of relative abundance, and therefore the abundance of protein containing this pair of compounds can also be measured.

In this study, we used a similar approach to quantify contaminating transition metal complexes, where $^{13}C/^{2}H$ chelator–amino acid conjugates were added to a radio-metal solution. The amounts of contaminating transition metal complexes were quantified by LC-MS analysis using the corresponding light metal complexes containing only native $^{12}C/^{1}H$ as internal standards. Based on the intensities of the pairs of metal complexes, non-radioactive metal contaminants present in metal radionuclide formulations can be readily quantified for determination of the ESA for the specific chelator.

To demonstrate this new technology, we synthesized $^{12}C/^{1}H$- and $^{13}C/^{2}H$-labelled agents of EdF-DOTA (Ethylenediamine-F(Phe)-DOTA, Fig. 1), and used them to quantify non-radioactive metal impurities in solutions of the PET radionuclide, Cu-64 ($T_{1/2}$ = 12.7 h, β^{+}: 0.655 MeV, 17.8%; β^{-}: 0.573 MeV, 41%).

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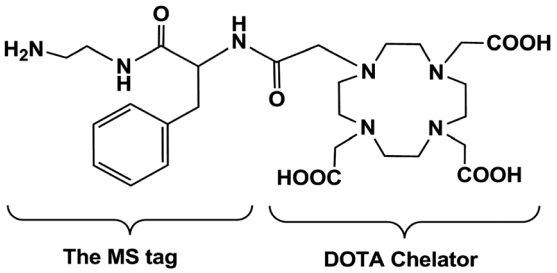

Fig. 1 Structure of EdF-DOTA consisting of a MS tag and DOTA chelator.

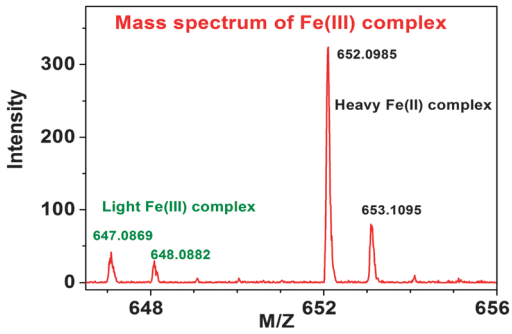

Fig. 2 MS spectrum of Fe(III)-DOTA-EdF complexes.

The use of the $^{13}C/^{2}H$ MS tag was inspired by a recent report on the use of deuterium-labelled reagents for protein quantification,[19] as the cost of a $^{13}C/^{2}H$-encoded agent is significantly less expensive than a fully encoded ^{13}C-labelled compound. The two reagents are a set of structurally identical molecules consisting of a MS tag (moieties that can be easily identified by MS) and a metal chelation group. The "heavy" MS tag is encoded with $^{13}C_2$ (50 atom% ^{13}C in phenylalanine) and $^{2}H_4$ (98 atom% D in ethylenediamine), and the "light" MS tag includes only native $^{12}C/^{1}H$. DOTA was chosen as the metal chelation group since it is widely used in metal-based radiopharmaceuticals and chelates with the transition metals Fe^{3+}, Ni^{2+}, Cu^{2+}, Co^{2+}, and Zn^{2+} that are the major impurities in cyclotron-produced Cu-64.[20] In order to simplify the quantification process, a complete separation between MS peaks from the heavy and light complexes (at least 5 Da) is required. For example, the MS of a Ni^{2+} complex of light EdF-DOTA is distributed from 650 to 654 due to naturally occurring nickel (Ni), which is primarily composed of four stable isotopes, ^{58}Ni, ^{60}Ni, ^{61}Ni and ^{62}Ni. In order to minimize the overlap between the light and heavy Ni complex, the MS of a Ni(II) complex with the heavy reagent should have a MW of at least 655, which is a 5 Da (or more) difference between the heavy and light reagents (Fig. S1, ESI†).

The heavy EdF-DOTA agent was prepared by conventional solid-phase synthesis (Scheme S1, ESI†). Firstly, ^{2}H enriched ethylenediamine was loaded onto the trityl chloride resin *via* its primary amine group. Next, Fmoc-($^{13}C_2$)Phe-OH that was prepared from the $^{13}C_2$-enriched phenylalanine was attached to the resin using HBTU/HOBt as the coupling reagents. After the Fmoc group was removed, a primary amine group was generated for the subsequent DOTA–NHS coupling. Finally, the EdF-DOTA agent with the heavy tag was cleaved from the resin by acid treatment and purified by HPLC. The light-tagged agent was synthesized in a similar manner using native ethylenediamine and phenylalanine. After obtaining the purified light EdF-DOTA reagent, internal standards of transition metal complexes (10 μM) were prepared by incubating excess light EdF-DOTA with a known amount of the transition metals for 30 min at 60 °C. The complexes are stored at −80 °C.

To prove that the above five transition metals do not bind with the MS tag, the MS tag without DOTA was prepared and incubated with those transition metals under the radiolabelling conditions. Based on HPLC and MS analysis, there was only the MS tag in the incubation mixture and no metal complex was observed. To further demonstrate the capability to quantify metal chelates by LC-MS, a heavy Fe^{3+} complex solution (Fe(III)-DOTA-EdF; 0.5 nmol) was mixed with the light Fe^{3+} complex solution (0.1 nmol), and diluted with NH_4OAc buffer for LC-MS analysis (Fig. 2). By comparing the total ion counts between heavy and light Fe(III)-DOTA-EdF complexes, the ratio of heavy (Fe(III)) to light (Fe(III)) complexes was 401/82 = 4.89, which is very close to the predetermined ratio of 5.0. The LC-MS approach was also used to measure the ratios of heavy and light Cu(II) complexes that were predetermined from 0.1 to 6.0, and a good linear correlation (R = 0.9934) between the predetermined ratio and LC-MS ratio was demonstrated over a broad range (Fig. S2, ESI†).

Following the above validation of the LC-MS method, the light and heavy EdF-DOTA agents were used to quantify the metal impurities in a solution of cyclotron produced Cu-64 from one of the two vendors in the U.S. The Cu-64 solution was neutralized and buffered with 0.1 M NH_4OAc solution (pH = 6.80), and when it was completely decayed (1 week), the excess heavy EdF-DOTA agent was added and incubated at 22 °C for 10 or 30 min, or at 60 °C for 30 min. After the incubation, the light complex solutions (internal standards) were added and mixed thoroughly. The mixture was then loaded into the LC-MS for analysis. Under the optimized LC-MS conditions, the five complexes were completely separated (Fig. 3). The UV trace was not shown here because the low molar absorptivity of the complex solutions led to very low signals. The mole amounts of the heavy metal complexes were calculated by comparing the MS

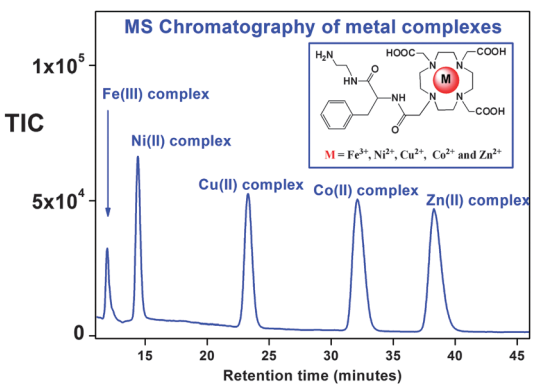

Fig. 3 TIC trace of LC-MS chromatography of the light–heavy complex mixtures.

Table 1 Heavy complexes and calculated ESA at 60 °C for 30 min

Metal complex	Fe(III)	Ni(II)	Cu(II)	Co(II)	Zn(II)
Standard intensity	726 ± 13	2987 ± 203	2405 ± 219	2148 ± 210	734 ± 10
Sample intensity	1943 ± 134	2299 ± 131	1440 ± 211	345 ± 9	2537 ± 101
Sample (pmol)	133	39	30	8	172
Effective SA (GBq μmol^{-1})	19.4 = 7.4 MBq/(133 + 39 + 30 + 8 + 172) pmol				

Table 2 Heavy complexes and calculated ESA under different conditions

Complex (pmol)	Fe(III)	Ni(II)	Cu(II)	Co(II)	Zn(II)	ESA (GBq μmol^{-1})
22 °C, 10 min	37	30	23	11	120	33.5
22 °C, 30 min	48	32	25	7.5	152	28.0
60 °C, 30 min	133	39	30	8	172	19.4

intensities between the heavy and light complexes to the known amount of the light complex (50 pmol). The effective specific activities were then calculated based on the Cu-64 activity (7.4 MBq (200 μCi), decay corrected to the time of receipt) and the total mole amount of five heavy complex contaminants (382 pmol).

The LC-MS measurements were performed in triplicate and the results obtained at 60 °C with 30 min incubation are summarized in Table 1. Only 7.4 MBq of Cu-64 was needed to obtain adequate TIC chromatograms, which demonstrates the high sensitivity of this LC-MS approach. It should be noted that although we allowed the Cu-64 to decay prior to analysis, radioactive samples are expected to give the same results. The major metal impurities in the test solutions were confirmed to be Fe^{3+}, Ni^{2+}, Cu^{2+}, Co^{2+} and Zn^{2+}; there were no other MS peaks in the TIC chromatogram. The metal-complex contaminants were quantified, and the results indicate that the Zn^{2+} and Fe^{3+} dramatically affected the radiolabelling of EdF-DOTA with the cyclotron produced Cu-64. Therefore, purification of Fe^{3+} and Zn^{2+} should be a focus for secondary purification of Cu-64. Alternatively, developing a chelator that specifically chelates Cu^{2+} with minimal Fe^{3+} and Zn^{2+} chelation is highly desired for improving ESA of Cu-64 radiopharmaceuticals.

This LC-MS method also allows for optimization of the labelling conditions to achieve high specific activity by using conditions favouring the chelation of a specific radio-metal. From the data shown in the Table 2, it was observed that the ESA varied with different incubation times and temperatures. Extending the incubation time or increasing the incubation temperature resulted in decreased ESA. At elevated temperatures, significantly more Fe^{3+} and Zn^{2+} chelated EdF-DOTA, whereas only slightly more Cu^{2+} complexed the EdF-DOTA reagent, resulting in the ESA decreasing from 28.0 GBq μmol^{-1} at 22 °C to 19.4 GBq μmol^{-1} at 60 °C, and after decay correction, it was close to the titration result from vendor. A similar result was obtained with extended incubation time (10 to 30 min), and the ESA decreased from 33.5 GBq μmol^{-1} to 28.0 GBq μmol^{-1}. These data demonstrate that this LC-MS approach can be used to optimize radiolabelling conditions to decrease chelation of contaminating metals for achieving higher ESA, since it can be challenging to separate DOTA-conjugated peptides that are complexed with Cu^{2+} in the presence of other contaminating metals, such as Fe^{3+}, Zn^{2+}.

In summary, a novel LC-MS approach has been developed to quantify non-radioactive metal impurities and determine effective specific activity (ESA) of radio-metals. $^{13}C/^{2}H$- and $^{12}C/^{1}H$-labelled EdF-DOTA agents were synthesized and were used for rapid and sensitive quantification of metal impurities found in cyclotron produced Cu-64. Besides the quantification of radio-metals for ESA, this LC-MS method can also be used as a valuable tool to optimize radiolabelling conditions and evaluate the specificity of a chelator for a particular radio-metal. Achieving high ESA for radio-metals is critical for PET imaging or targeted radiotherapy of low capacity receptor systems. This novel methodology can be applied for a variety of radio-metals and chelators in the development of PET imaging radiopharmaceuticals and metal-based targeted radiotherapy agents for many diseases, including cancer, cardiovascular disease and pulmonary disorders.

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