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Highly sensitive antibody-free μ LC–MS/MS quantification of rhTRAIL in serum

Background: We describe an antibody-free approach to quantify rhTRAIL^{WT} (wild-type) and its closely related death receptor 4 selective variant rhTRAIL^{4C7} in human and murine serum by multiplex LC–MS/MS on a microfluidics interface. **Methodology:** Enrichment of rhTRAIL was performed by strong cation-exchange (SCX) followed by immobilized metal affinity (IMAC) solid-phase extraction. This was followed by trypsin digestion and using methionine-containing signature peptides after fully oxidizing the methionine residue with 0.25% (w/w) hydrogen peroxide. **Conclusion:** Absolute quantification was reaching down to 0.5 ng/ml for rhTRAIL^{WT} (8.5 pM) and 2 ng/ml for rhTRAIL^{4C7} (34 pM) in 100 μ l human serum. To support preclinical studies in mice, the analysis was optimized further, for a sample volume of 20 μ l murine serum.

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Keywords: ¹⁵N-metabolically labeled internal standard • antibody-free • immobilized metal affinity (IMAC) enrichment • quantitative LC–MS/MS • strong cation exchange (SCX) enrichment • TNF-related apoptosis-inducing ligand (TRAIL)

There is an increasing interest in the quantitative determination of proteins in biological matrices with LC–MS/MS as a complement or alternative to the widely used ligand-binding assays (LBAs; e.g., ELISA) [1–3]. LC–MS/MS analysis is generally based on the quantitation of signature peptides from proteolytic digests of the proteins of interest [4]. To achieve a comparable sensitivity to LBAs in complex biological matrices, sample preparation plays a crucial role [5]. In fact, pg/ml to low ng/ml sensitivity for large proteins often requires immunoaffinity enrichment at the protein or peptide level, partially alleviating the advantage of LC–MS/MS over LBAs [6–10]. Ideally, enrichment should be performed at the protein level to avoid generating a myriad of highly similar peptides, notably from high-abundance proteins, resulting in reduced selectivity and sensitivity [11]. To take full advantage of the LC–MS/MS approach, we previously developed an

antibody-free approach using immobilized metal affinity chromatography (IMAC) for recombinant human TNF-related apoptosis-inducing ligand (rhTRAIL) in serum [12]. This approach permitted quantification of rhTRAIL, consisting of the extracellular TRAIL domain (amino acids 114–281 of sequence P50591) and the DR4-specific rhTRAIL^{4C7} variant in human and mouse serum with a lower limit of quantitation of 20 ng/ml.

Wild-type rhTRAIL and the death-receptor-specific rhTRAIL variants, like rhTRAIL^{4C7}, are promising novel anti-cancer biotherapeutics which restore apoptosis in tumor cells due to controlled, programmed cell death with minimal cytotoxic effects on healthy tissues [13–15]. The presence of decoy receptors was the presumed reason that efficacy of chemotherapy was not improved in Phase I and II studies of patients treated with rhTRAIL (Dulanermin) [16–21]. A way to improve effi-

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cacy is the design and optimization of DR4- and DR5-specific rhTRAIL variants, which show promising results in cancer cells (*in vitro*) and in tumor-bearing animals (*in vivo*) [22–25]. However, in order to be able to develop such variants, it is necessary to have variant-specific quantitative bioanalytical methods.

In this paper we describe the further development of an antibody-free sample preparation by combining IMAC with strong-cation-exchange (SCX) into a 2D-SPE enrichment approach improving sensitivity close to the current level of a validated ELISA [17]. Due to the more efficient clean-up, it was possible to select two signature peptides for each protein from which one was used for quantification and the other for confirmation [26]. Adapting this approach to a microfluidics-based LC–MS/MS system allowed us to reach low ng/ml sensitivity with only 20 µl serum making repeat sampling from mice in preclinical studies possible.

Experimental section

Chemicals & protein standards

Water was purified by a Milli-Q® Advantage A10® System (conductivity 18.2 MΩ/cm, Millipore, Billerica, MA, USA). J.T.Baker® LC–MS grade acetonitrile and methanol were obtained from Avantor Performance Materials (PA, USA). Sodium phosphate dibasic dihydrate and sodium dihydrogen phosphate monohydrate, formic acid, glycerol, Tween® 20, sodium chloride, imidazole, hydrogen peroxide, and trypsin from porcine pancreas (Type IX-S) were purchased from Sigma Aldrich (MO, USA). Human serum (male, individual) was obtained from Sera Lab (Hayward's Heath, UK). Murine serum (strain BALB/cOlaHsd, female, individual) was obtained from Harlan Laboratories (Horst, The Netherlands). The protein standards, rhTRAIL^{WT} and rhTRAIL^{4C7} with a concentration of 0.83 and 1.38 mg/ml, respectively, and the labeled protein standards, ¹⁵N-rhTRAIL^{WT} and ¹⁵N-rhTRAIL^{4C7} with a concentration of 0.86 and 0.23 mg/ml, respectively, were expressed and purified as described in Reis *et al.* [27] and Wilffert *et al.* [12]. ¹⁵N-labelled proteins were produced in *E.coli* grown on ¹⁵N-labelled ammonium acetate as only nitrogen source. All protein standards were stored in aliquots at –80°C. Two internal standard working solutions were prepared for quantitation: one in human serum containing 110 ng/ml ¹⁵N-rhTRAIL^{WT} and 550 ng/ml ¹⁵N-rhTRAIL^{4C7} and another one in murine serum containing 150 ng/ml ¹⁵N-rhTRAIL^{WT} and 750 ng/ml ¹⁵N-rhTRAIL^{4C7}.

Strong cation exchange & immobilized metal affinity chromatography solid-phase extraction

Strong cation exchange (SCX) or IMAC solid phase extraction (SPE) resins were separately prepared as

follows. Two-hundred µl of SCX-resin (TSKgel® SP-3PW, slurry in 20% (v/v) ethanol in water, methacrylic polymer-based particles, particle size 30 µm, 250 Å, TOSOH Bioscience, Tokyo, Japan) were transferred to a 1.5 ml tube. 1 ml of methanol was added to the slurry and vortexed, which was followed by centrifugation at 1500 g for 3 min. The supernatant was removed and replaced by 1 ml of SCX buffer pH 7.5 (20 mM phosphate buffer, 10% glycerol, 0.005% Tween® 20) containing 40 mM NaCl to equilibrate the SCX resin. The SCX resin was recovered by centrifugation at 1500 g for 3 min followed by another addition of 700 µl (for human serum samples) or 790 µl (for murine serum samples) SCX buffer pH 7.5 containing 40 mM NaCl.

IMAC enrichment was performed with 100 µl Ni²⁺ resin (Ni Sepharose™ 6 fast flow, slurry in 20% (v/v) ethanol in water, 6% cross-linked agarose, particle size 90 µm, GE Healthcare, Freiburg, Germany) which was transferred to a 1.5 ml tube. 500 µl of water were added to the slurry, which was followed by vortex-mixing and centrifugation at 1500 g for 3 min. The water was removed and replaced by 500 µl IMAC buffer pH 8 (20 mM phosphate buffer, 10% glycerol, 0.005% Tween® 20) containing 15 mM imidazole to equilibrate the IMAC resin. After vortexing the resin was sedimented by centrifugation at 1500 g for 3 min and 500 µl IMAC buffer pH 8 containing 15 mM imidazole were added.

SCX-IMAC enrichment of rhTRAIL

Ten µl of human serum internal standard working solution were added to 100 µl human serum to give a final concentration of 10 ng/ml of ¹⁵N-rhTRAIL^{WT} and 50 ng/ml of the variant ¹⁵N-rhTRAIL^{4C7}. Ten µl of murine serum internal standard working solution were added to 20 µl murine serum to give a final concentration of 50 ng/ml of ¹⁵N-rhTRAIL^{WT} and 250 ng/ml of the variant ¹⁵N-rhTRAIL^{4C7}. One hundred µl of human serum or 20 µl of murine serum were added to the previously prepared SCX resin (in SCX buffer pH 7.5 containing 40 mM NaCl) and mixed on a rotating wheel for 10 min at a speed of 20 rpm. The suspension was transferred to an empty solid-phase extraction (SPE) cartridge (volume 1 ml, Agilent, CA, USA) that was placed in a Cerex Positive Pressure SPE processor (Varian, CA, USA). Positive air pressure was applied to the suspension in the SPE cartridge, followed by placing a frit on top of the resin. The SCX resin was washed with 1 ml SCX buffer pH 7.5 containing 40 mM NaCl and eluted with 400 µl SCX buffer pH 8.0 containing 100 mM NaCl and 15 mM imidazole. The final elution volume was directly collected in the tube containing the prepared IMAC resin (see above).

This suspension was mixed for 10 min on a rotating wheel (diameter 270 mm) at a speed of 20 rpm, transferred to an empty SPE cartridge and the IMAC resin was washed with 500 μ l IMAC buffer pH 8.0 containing 100 mM NaCl and 15 mM imidazole and eluted with 200 μ l IMAC buffer pH 8.0 containing 100 mM NaCl, 150 mM imidazole and 20% acetonitrile. For each step, positive air pressure was applied until the last drop was removed from the resins. For each sample, fresh SCX and Ni²⁺-resins and empty SPE cartridges were used. Recovery of SCX, IMAC and SCX-IMAC enrichment was determined by comparing blank sera spiked with the same concentration of rhTRAIL before and after enrichment.

Trypsin digestion & oxidation of signature peptides

Ten μ l of 50 μ g/ml trypsin was added to 200 μ l of the SCX-IMAC enriched sample resulting in an approximate enzyme-to-substrate ratio (w/w) of 1:10. Proteins were digested by incubation at 37°C with vortex-mixing at 450 rpm for 2 h in a Thermomixer comfort (Eppendorf, Hamburg, Germany). The digestion was stopped by adding 10 μ l of 23% formic acid. This was followed by adding 10 μ l of 11.5% hydrogen peroxide to fully oxidize the methionine residue in the signature peptides for 1 min at room temperature. 20 μ l were used for LC–MS/MS analysis.

Determination of total protein concentration

The percentage protein removal was determined by comparing the total protein concentration of the collected SCX, IMAC and SCX-IMAC elution fractions with the initial serum sample. All samples were diluted with PBS (phosphate-buffer saline; 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) to fall within the calibration range of the micro bicinchoninic acid (microBCA) assay. Bovine serum albumin was used as calibration standard and the assay was performed according to the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL, USA). A Fluostar Optima plate reader (BMG Labtech, Ortenberg, Germany) was used for measuring absorbance at 580 nm.

Gel electrophoresis & silver staining

To identify remaining matrix proteins before and after SCX, IMAC and SCX-IMAC enrichment, the collected fractions were analyzed by gel electrophoresis in the presence of SDS. Prior to gel electrophoresis, the collected fractions, rhTRAIL as standard and the protein marker (Novex® Sharp Unstained Protein Standard) were diluted with PBS. 20 μ l were loaded on a precast polyacrylamide gradient gel (8 cm $\times$ 8 cm,

NuPAGE® Novex® 4–12% Bis-Tris gel), which was run with an SDS-containing running buffer (pH 7.7, NuPAGE® MES SDS Running buffer) according to the manufacturer's instructions (Thermo Fisher Scientific, MA, USA). All gel electrophoresis products were purchased from Life Technologies (Thermo Fisher Scientific). Silver staining of the gels was done as described by Yan *et al.* [28].

In-gel trypsin digestion & nanoLC-QTOF MS analysis for protein identification

Protein bands were excised from the polyacrylamide gel, destained and in-gel digested as described by Franciosi *et al.* [29]. Digests were analysed by nanoLC-QTOF MS using an UltiMate 3000 Rapid Separation LC (RSLC) system (Thermo Fisher Scientific, Waltham, MA, USA) linked to a Maxis II™ QTOF mass spectrometer (Bruker, Billerica, MA, USA) (see [Supplementary Table 1 & 2](#) for the conditions of the nanoLC-QTOF MS analysis as well as for details about the database search). Protein identifications were considered significant if at least two unique peptides were matched to the obtained MS/MS spectra with a false discovery rate of 1% on the protein level and of 5% on the peptide level.

(Micro) LC–MS

Chromatographic separation for comparing the different enrichment methods (SCX, IMAC and SCX-IMAC) was performed using an Acquity I-Class UPLC system (Waters, Milford, MA, USA). Peptides were separated on a CSH C₁₈ column (150 $\times$ 2.1 mm, 1.7 μ m, 130 Å) at 50°C and a flow rate of 0.4 ml/min. Gradient elution was performed with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). 20 μ l of the enriched sample (autosampler at 10°C) were injected, the gradient started at 3% B and linearly increased with 2.5% B/min for 9.5 min. The column was cleaned for 2 min at 90% B and equilibrated resulting in a total run time of 15 min. LC–MS/MS for the quantitative analysis of the signature peptides of rhTRAIL^{WT} and the rhTRAIL^{4C7} variant was downscaled by using an Acquity M-Class nanoUPLC system (Waters, Milford, MA, USA). The LC system was equipped with a microfluidics iKey UPLC CSH C₁₈ column (100 $\times$ 0.15 mm, 1.7 μ m, 130 Å) running at 40°C and a flow rate of 3 μ l/min. 20 μ l of the enriched sample were trapped at a flow rate of 20 μ l/min for 2.5 min at 3% B on a Trap Symmetry C₁₈ column (50 $\times$ 0.3 mm, 5 μ m, 100 Å). The trapped sample was transferred and separated by switching the iKey online and performing gradient elution starting at 3% B, linearly increasing with 1.5% B/min for 17 min. Hereafter, the column was cleaned

for 2 min at 90%B and equilibrated, which resulted in a total run time of 24 min.

A Waters Xevo TQ-S triple quadrupole mass spectrometer was used for detection and an IonKey source (Waters, Milford, MA, USA) was used for connecting the microLC. Electrospray ionization was done in positive (ESI⁺) mode. For LC separation at a flow of 0.4 ml/min the following source parameters were used: source temperature 150°C, desolvation temperature 500°C, capillary voltage 3.00 kV, cone voltage 50 V, desolvation gas (nitrogen) 1000 l/h, cone gas (nitrogen) 150 l/h, collision gas (argon) 0.18 ml/min and nebulizer gas pressure 7.0 bar. For μ LC separation at a flow of 3 μ l/min, the following source parameters were used: capillary voltage was increased to 3.50 kV, cone gas was decreased to 20 l/h and the desolvation temperature and desolvation gas flow were turned off.

Selected SRM transitions and collision energies for the signature peptides of rhTRAIL^{WT}, ¹⁵N-rhTRAIL^{WT}, rhTRAIL^{4C7} and ¹⁵N-rhTRAIL^{4C7} are shown in the [Supplementary Information \(Supplementary Table 3\)](#). For each protein, two different signature peptides were selected one of which was used for quantification, and the second for confirmation. The following peptides were selected for quantification: YTSYPDPILLM(Ox)K for rhTRAIL^{WT} and YTDYPDPILM(Ox)K for rhTRAIL^{4C7}, containing an amino acid mutation from serine to aspartic acid at position 3 for rhTRAIL^{4C7}. For confirmation, the following peptides were selected: SGHSFLSNLHLR for rhTRAIL^{WT} and GHSFLSNLHLR for rhTRAIL^{4C7} containing an amino acid mutation from serine to arginine at position 1 for rhTRAIL^{4C7}. Results were accepted if the ratio between the peak areas of the two peptides were within $\pm 25\%$ of the mean ratio.

Results & discussion

We previously reported a validated LC–MS/MS method to quantify rhTRAIL^{WT} and the death receptor 4-specific variant rhTRAIL^{4C7} in human and murine serum supporting preclinical PK studies in mice [12]. Ni²⁺-IMAC was selected for enrichment, because rhTRAIL contains a number of surface-exposed histidines [30]. However, application of this method required 100 μ l serum, which means that one mouse had to be sacrificed per time point of the PK time curve. In this article, we describe the further development of this approach to increase sensitivity from 20 to 0.5 ng/ml and to reduce sample requirement from 100 to 20 μ l [12].

SCX, IMAC & SCX-IMAC protein enrichment

To select additional sample preparation steps in a rational manner, we separated the major remaining matrix proteins after IMAC by SDS-PAGE ([Figure 1](#), lane 4 of

the IMAC panel) and identified them by excising the corresponding bands from the gel followed by in-gel trypsin digestion, LC–MS/MS on a quadrupole time-of-flight (QTOF) mass spectrometer and database search ([Supplementary Table 4](#)). We found that the isoelectric point (pI) of most remaining serum proteins in the IMAC fraction was considerably lower than the pI of rhTRAIL (pI 8.9) (see [Supplementary Table 4](#)). Based on these results, we reasoned that an additional sample pretreatment step with SCX at pH 7.5 should discriminate between rhTRAIL (expected to bind) and most of the serum proteins (expected not to bind). Since SCX enrichment was performed at the protein level (rhTRAIL ≈ 58.5 kDa, pore size of the resin 250 Å), it was necessary to let proteins interact with the beads for 10 min on a rotating wheel for binding. Adding 40 mM sodium chloride during loading and washing resulted in removal of most remaining serum proteins as illustrated by SDS-PAGE analysis ([Figure 1](#), lanes 2–3 of the SCX panel). rhTRAIL was finally eluted with 100 mM sodium chloride at pH 8.

Compared to IMAC, SCX showed a slightly better clean-up as indicated by total protein determination in the eluted fractions of both enrichment methods (SCX clean-up ca. 99%, IMAC clean-up ca. 95%). Visual comparison of the SCX and IMAC elution fractions after SDS-PAGE ([Figure 1](#), lanes 4 of SCX and IMAC panels) also indicated a slightly improved cleanup by SCX, while LC–MS/MS analysis of rhTRAIL^{WT} at 10 ng/ml (initial serum concentration) showed comparable sensitivity and a comparable recovery (SCX 72%, IMAC 71%) [30]. Combining both enrichment steps in an SCX-IMAC sequence resulted in the removal of >99.9% of total protein with a total recovery of 49% for rhTRAIL. Comparing the identified remaining serum proteins in the eluates of the IMAC and the SCX steps underscores the orthogonality of both methods ([Supplementary Table 4](#)), since for example β -2-glycoprotein with a pI of 8.3 is retained by SCX but not IMAC while apolipoprotein A-I (pI 5.6) is retained by IMAC but not SCX. The most abundant serum protein, albumin (pI 5.3) is partially retained by both SCX and IMAC indicating other modes of interaction, but the combination of SCX-IMAC led to the efficient removal of albumin.

Downscaling the LC separation

By reducing the column diameter from 2.1 mm to 150 μ m using a microfluidics system, we were able to reduce the volume of mouse serum from 100 μ l to 20 μ l without compromising overall sensitivity due to more efficient electrospray ionization at the reduced flow rate of 3 μ l/min (instead of 400 μ l/min) [12]. One might expect that reduction would be more significant considering that the flow rate is more than 100-

Figure 1. SDS-PAGE analysis of 100 μ g/ml rhTRAIL^{WT} in human serum after SCX, Ni²⁺ IMAC and the combination of SCX-IMAC enrichment. Lanes: M, molecular weight marker; T, 25 μ g/ml of rhTRAIL^{WT} in buffer (500 ng in 20 μ l of loading volume) for all gels. SCX: Lane 1: human serum (100x diluted), lane 2: flow through with 40 mM NaCl (200x diluted), lane 3: wash with 40 mM NaCl (10x diluted); lane 4: elution with 100 mM NaCl (4x diluted); lane 5: elution with 500 mM NaCl (4x diluted). IMAC: lane 1: human serum (100x diluted), lane 2: flow through with 15 mM imidazole (200x diluted), lane 3: wash with 15 mM imidazole (10x diluted); lane 4: elution with 150 mM imidazole (4x diluted); lane 5: elution with 500 mM imidazole (4x diluted). SCX-IMAC: lane 1: human serum (100x diluted), lane 2: flow through with 15 mM imidazole (10x diluted), lane 3: wash with 15 mM imidazole (5x diluted); lane 4: elution with 150 mM imidazole (2x diluted); lane 5: elution with 500 mM imidazole (2x diluted). A loading volume of 20 μ l was used in all lanes and all protein bands in the elution steps of lane 4 of all enrichment methods were identified by in-gel trypsin digestion and LC-QTOF MS/MS analysis (Supplementary Table 4). The arrow indicates the position of rhTRAIL^{WT} at 19.5 kDa. The rhTRAIL trimer dissociates into three identical monomers upon analysis by SDS-PAGE. IMAC: Immobilized metal affinity; SCX: Strong cation-exchange.

times lower, but unfortunately peak width was also increased from ≈ 0.15 min (400 $\mu\text{l}/\text{min}$) to ≈ 0.45 min (3 $\mu\text{l}/\text{min}$). Still, requiring only 20 μl of serum alleviates the need to sacrifice a mouse for each time point of a PK study, as multiple samples can be taken from the same animal. Figure 2 shows the LC–MS/MS chromatograms of both signature peptides in murine serum for rhTRAIL^{WT} and rhTRAIL^{4C7} at the lower

limits of quantitation (LLOQ) of 2 ng/ml and 10 ng/ml, respectively.

The efficient enrichment of rhTRAIL by SCX-IMAC is a prerequisite to take full advantage of the μLC column with its limited loading capacity (~ 10 μg of protein digest). Using 100 μl of (human) serum (~ 8 mg total protein in the starting material) improved sensitivity to 0.5 ng/ml for rhTRAIL^{WT} and to 2 ng/ml

Figure 2. μLC –MS/MS analyses of rhTRAIL^{WT} and rhTRAIL^{4C7} in 20 μl mouse serum at 2 ng/ml and 10 ng/ml, respectively. The corresponding blank sera are shown in red and the amino acid changes between rhTRAIL^{WT} and rhTRAIL^{4C7} are underlined.

Figure 3. μ LC–MS/MS analysis of rhTRAIL^{WT} (signature peptide YTSYPDPILLM(Ox)K) and rhTRAIL^{4C7} (signature peptide YTDYPDPILLM(Ox)K) in 100 μ l human serum at 0.5 and 2 ng/ml. The corresponding blank sera are shown in red.

for the rhTRAIL^{4C7} variant (Figure 3). The change in order of elution is likely due to a matrix effect, which is different for murine and human serum. These concentrations show a 40 times increase in sensitivity for rhTRAIL^{WT} and a ten times increase for rhTRAIL^{4C7} compared with our previously developed antibody-free method in human serum [12]. On top of that these concentrations come also close to the sensitivity of a reported ELISA (LLOQ of 0.2 ng/ml) used to support a clinical study of rhTRAIL [17]. To the best of our knowledge, this is the first report of sub-ng/ml sensitivity for a protein of a size >50 kDa, quantified in serum with LC–MS/MS without antibody-based extraction.

Despite the fact that the volume of human serum is not a limiting factor, the use of more than 100 μ l of human serum will likely not result in an increase in sensitivity. This is due to the approximately 8 μ g of protein digest left after SCX-IMAC enrichment, which is already close to the maximum loading capacity of approximately 10 μ g of protein digest of the μ LC column.

Method validation & suitability for studying the PK of rhTRAIL^{WT} & rhTRAIL^{4C7} in mice

The described method was validated with respect to linearity, accuracy, precision, selectivity and the stability of rhTRAIL^{WT} and rhTRAIL^{4C7} and applied to support a PK study in mice [31,32]. Linearity of the method was assessed for rhTRAIL^{WT} between 2 ng/ml and

2000 ng/ml and for rhTRAIL^{4C7} between 10 ng/ml and 10,000 ng/ml. The calibration curve (weighting factor $1/x^2$) of rhTRAIL^{WT} (Supplementary Figure 1) and rhTRAIL^{4C7} (Supplementary Figure 2) consisted of eight concentration levels all within $\pm 15\%$ bias and 15% CV ($\pm 20\%$ bias and 20% CV at the LLOQ level) and correlation coefficients (r^2) were all above 0.9997. Quality control (QC) samples of rhTRAIL^{WT} and rhTRAIL^{4C7}, respectively, were prepared at three different concentration levels: 6/30 ng/ml (QC low); 80/400 ng/ml (QC medium); 1600/8000 ng/ml (QC high). Within-run and between-run accuracy and precision (Supplementary Table 5) were evaluated at these three quality control levels as well as at the LLOQ and were all within $\pm 15\%$ bias and 15% CV ($\pm 20\%$ bias and 20% CV at the LLOQ level). Stability of rhTRAIL^{WT} and rhTRAIL^{4C7} (Supplementary Table 6, top panel) was assessed at the protein level during bench-top storage (30 h) and while undergoing freeze/thaw cycles (5 $\times$) and at the peptide level during autosampler storage (24 h at 10°C). All stability experiments demonstrated a bias within $\pm 15\%$ and a CV within 15% at the QC low and QC high levels. Between-matrix variability (selectivity) was validated (Supplementary Table 6, bottom panel) in six different individual murine serum samples which were analyzed blank and spiked at the LLOQ level. The blank levels revealed no interferences and the accuracy and precision at the LLOQ level were all within $\pm 20\%$ bias and 20% CV.

Figure 4. PK profiles in mice which received a simultaneous dose of 5 mg/kg rhTRAIL^{WT} and 5 mg/kg rhTRAIL^{4C7} by a intraperitoneal injection. The samples above the upper limit of quantitation of 10,000 ng/ml were analyzed by first 100 times diluting the serum samples with SCX loading buffer. Samples have also been previously analyzed and used in [12].

Figure 4 shows the suitability of the improved and validated μ LC–MS/MS method for rhTRAIL^{WT} and rhTRAIL^{4C7} by reanalysis of (20 μ l) serum samples from a previously performed PK study in mice that were i.p. administered simultaneously with 5 mg/kg of rhTRAIL^{WT} and rhTRAIL^{4C7} [12].

Conclusion

There are doubts as to whether antibody-free LC–MS/MS methods might be able to reach the same sensitivity as immunoassays, notably sandwich ELISAs. Here we show that an LC–MS/MS method can reach similar LLOQs as a sandwich ELISA that has been previously used to support a clinical study of rhTRAIL. This is due to the rational design of the sample preparation steps based on structural knowledge of the analyte (surface-exposed histidines) and the detailed analysis of the physicochemical properties of the co-enriched serum proteins versus those of rhTRAIL (isoelectric point). Miniaturization of

the LC system permitted analysis of rhTRAIL at the sub-ng/ml level and allowed discriminating between rhTRAIL^{WT} and the rhTRAIL^{4C7} variant in a single run. Miniaturization had the additional benefit of being able to reduce the required amount of serum to 20 μ l while maintaining an LLOQ for rhTRAIL^{WT} of 2 ng/ml and of 10 ng/ml for the variant rhTRAIL^{4C7}, largely sufficient for preclinical and clinical PK studies. rhTRAIL enrichment was performed by orthogonal SCX-IMAC solid-phase extraction resulting in >99.9% protein removal. To provide absolute quantification, ¹⁵N-metabolically labeled rhTRAIL^{WT} and the rhTRAIL^{4C7} variant were used as internal standards, and the methionine-containing signature peptides were fully oxidized to the sulfoxide with hydrogen peroxide as previously described [12].

Future perspective

This example sets the stage for developing additional cytokine assays using antibody-free workflows and LC–MS/MS. It is to be expected that the sensitivity of targeted LC–MS/MS assays will increase further due to more advanced instrumentation and a growing knowledge base concerning tailored sample preparation approaches. Considering that development of specific antibodies is a lengthy procedure with an often uncertain outcome, we believe that antibody-free LC–MS/MS will play an increasing role in protein bioanalysis notably during the early stages of drug development.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/bio.16.30

Financial & competing interests disclosure

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Executive summary

- Absolute quantification was reaching down to 2 ng/ml for rhTRAIL^{WT} (34 pM) and 10 ng/ml for rhTRAIL^{4C7} (170 pM) in 20 μ l murine serum.
- Adapting this approach to a microfluidics-based LC–MS/MS system allowed us to reach low ng/ml sensitivity with only 20 μ l serum making repeat sampling from mice in preclinical studies possible.
- Comparing the identified remaining serum proteins in the eluates of the IMAC and the SCX enrichment underscored the orthogonality of both methods.
- The described method was validated in murine serum with respect to linearity, accuracy, precision, selectivity and the stability of rhTRAIL^{WT} and rhTRAIL^{4C7}.

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Papers of special note have been highlighted as:

• of interest; •• of considerable interest

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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