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Research Article

Quantification of total sBCMA in human plasma by peptide-level immunocapture LC-MS/MS

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ABSTRACT

Background: B-cell maturation antigen (BCMA) is a membrane protein that is overexpressed in multiple myeloma cells and can be targeted with biotherapeutic agents. BCMA is naturally shed by γ-secretase, leading to the formation of soluble BCMA (sBCMA), which circulates in the blood. sBCMA can affect the efficacy of BCMA-targeted therapies and act as a drug sink. Additionally, sBCMA can interfere with pharmacokinetic measurements when BCMA is directly targeted. Therefore, quantification of this biomarker during clinical trials is essential to assess the effective dose and understand pharmacokinetic results. When quantifying sBCMA using ligand binding assays or hybrid assays, the biotherapeutic can interfere with the capture of sBCMA, leading to an underestimation of its levels.

Methods: Samples were denatured, reduced, and alkylated prior to trypsin digestion. sBCMA peptide enrichment was performed using anti-peptide polyclonal antibodies. Reversed-phase chromatographic separation was conducted on a biocompatible C18 column with an analysis time of sixteen minutes per sample. The SCIEX QTRAP 5500 mass spectrometer operated in multiple reaction monitoring mode. The calibration curve was prepared by spiking recombinant sBCMA into monkey plasma.

Results: The parallelism between the authentic and surrogate matrices, as well as between the endogenous and recombinant proteins, was validated. Comparisons were made between protein and peptide level hybrid assays, with the peptide level approach effectively removing the interference of the biotherapeutic. Additionally, the peptide level immunocapture LC-MS/MS demonstrated ligand tolerance.

Conclusion: The peptide level immunocapture LC-MS/MS analysis eliminated the interference of anti-BCMA biotherapeutics, allowing for the quantification of total sBCMA in clinical samples while achieving a LLOQ of 10 ng/mL.

Introduction

B-cell maturation antigen (BCMA) is a transmembrane glycoprotein overexpressed in multiple myeloma (MM) cells, involved in the tumor survival and proliferation [1], with low levels in healthy cells. For these reasons, BCMA is a promising antigen for MM immunotherapy, and is currently targeted by biotherapeutics in development, including antibody drug conjugates (ADC) [2,3], T-cell [4,5] and natural killer (NK) cell engagers [6,7]. The ADC strategy is to target BCMA and internalize the cytotoxic conjugate, which leads to the proteolysis of the MM cell. The mechanism of action of T and NK cell engagers is to bring immune cells closer to MM cells by targeting BCMA and a membrane protein of T cells and NK cells, facilitating the formation of an immunological synapse to mediate target-specific killing of BCMA-positive cells by immune effector cells. BCMA expression is typically evaluated by immunohistochemistry and flow cytometry.

BCMA is naturally cleaved by γ-secretase [8], resulting in the production of soluble BCMA (sBCMA), a small protein with a short lifespan

Abbreviations: ACN, acetonitrile; ADC, antibody drug conjugate; APRIL, a proliferation inducing ligand; BAFF, B-cell activating factor; BCMA, B-cell maturation antigen; BLAST, Basic Local Alignment Search Tool; DTT, DL-Dithiothreitol; ESI, electrospray ionization; FA, formic acid; LBA, ligand binding assay; IAM, iodoacetamide; IS, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLOQ, lower limit of quantitation; MM, multiple myeloma; MRM, multiple reaction monitoring; MS, mass spectrometry; NK, natural killer; PBS, phosphate-buffered saline solution; QC, quality control; sBCMA, soluble B-cell maturation antigen; SDS, sodium dodecyl sulfate.

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circulating in the blood [9]. Levels of sBCMA in healthy individuals are around 20–40 ng/mL [10,11]. Elevated levels of sBCMA have been reported in MM patients compared to healthy individuals, and variations in concentration have been linked to treatment response [12,13]. The short half-life (24–36 h) of sBCMA in blood allows for a rapid turnover of the biomarker, making it a good assessment tool for detecting changes in the clinical status of MM patients [11].

sBCMA is a pharmacodynamic biomarker of the disease, but it is also an off-target of BCMA therapies, which could interfere with the mechanism of action of the biotherapeutic, reduce treatment efficacy, and impact pharmacokinetic measurements, including drug exposure. Higher levels of sBCMA have been associated with poorer outcomes and may require a higher dose of biotherapeutic [14]. Quantifying sBCMA is therefore essential for understanding the pharmacokinetics of the drug and anticipating a potential drug sink.

Ligand binding assays (LBAs) are preferred bioanalytical tools due to their high sensitivity. However, the small size of sBCMA (~5.4 kDa) reduces the number of immunogenic epitopes, complicating the development of sandwich assays. Moreover, as sBCMA is an off-target of the biotherapeutic, anti-BCMA therapies are suspected to interfere with the capture of sBCMA, which may affect the measurements.

Quantitative mass spectrometry (MS) has high specificity but lower sensitivity compared to LBAs. The most specific method for enriching the analyte of interest is affinity purification prior to MS analysis, as the immunocapture allows for increased sensitivity [15–17]. The affinity purification improves liquid chromatography (LC) performance, reduces ionization suppression in the MS source and enhances the performance of standard multiple reaction monitoring (MRM) methods. [18] Nowadays, hybrid assays are becoming more common in the analysis of biomarkers [19–22], as well as drug monitoring of therapeutic antibodies [23] and insulin analysis [24].

Hybrid assays including a protein level immunocapture were developed for the analysis of sBCMA [25,26]. However, in the context of BCMA therapy, this methodology may encounter the same drawbacks as the LBA. An alternative approach has been developed to overcome the biotherapeutic interference using an innovative peptide immunocapture LC-MS/MS method [15,27–29]. In this approach, the clinical sample is digested into peptides prior the immunocapture step with an anti-peptide antibody, and then analyzed by LC-MS/MS. This approach was multiplexed, automated for the quantification of human plasma proteins [30–32] and investigated for sBCMA in monkey plasma [25]. This approach could minimize the interference of the therapeutic drug and allows the quantification of total sBCMA in clinical samples.

This paper reports the development and validation of a peptide immunocapture LC-MS/MS analysis for the quantification of sBCMA in human plasma, with a lower limit of quantification (LLOQ) of 10 ng/mL. This LLOQ is sufficient for quantifying samples from healthy volunteers and patients. The interference from the pre-marketed Sanofi product and endogenous proteins was assessed and compared to the results obtained with a protein immunocapture LC-MS/MS method.

Material and methods

Reagents and materials

Human recombinant sBCMA was purchased from Abcam (Cambridge, United Kingdom). The biotinylated anti-peptide polyclonal rabbit antibody was custom-made and purchased from Agrobio (La Ferté Saint Aubin, France). The flanked internal standard (PyroQLRCSSNTPPL*TCQRYCN with L*: Leu [^{13}C6, ^{15}N]) was purchased from Sb-peptide (Saint Egrève, France).

K2-EDTA human plasma were purchased from Biopredic (Saint-Grégoire, France). K2-EDTA cynomolgus monkey plasma were purchased from Bioprim (Bazège, France).

Dynabeads™M280 Streptavidin beads were purchased from Invitrogen (Waltham, United States). The pierce trypsin was purchased from Thermo Scientific (Waltham, United States). DL-Dithiothreitol (DTT), iodoacetamide (IAM), sodium dodecyl sulfate (SDS) and ammonium bicarbonate (NH_4HCO_3) were purchased from Sigma-Aldrich (Saint-Louis, United States). Phosphate-buffered saline solution (PBS) was purchased from Gibco (Waltham, United States). Acetonitrile (ACN), ultrapure water, and formic acid (FA) were purchased from Fisher Scientific (Waltham, United States).

Anti-peptide immunocapture

Digestion

25 µL of sample (standards, QCs, blanks, and unknown samples) was diluted in 25 µL of NH_4HCO_3 (50 mM). 50 µL of 0.25 % (w/v) SDS solution was added and then the sample was mixed for five minutes at room temperature. 250 µL of NH_4HCO_3 (50 mM) at 5 ng/mL of flanked internal standard was added. 50 µL of DTT (100 mM) was added, and the samples were incubated at 60 °C for 30 min under gentle agitation. 50 µL of IAA (250 mM) was added, and the samples were incubated at 37 °C for 30 min under gentle agitation in the dark. After the addition of 250 µL of NH_4HCO_3 (50 mM) to dilute the reagents, 50 µL of trypsin at 160 µg/mL was added and the samples were incubated at 37 °C for 3 h under gentle agitation. At the end of the digestion, the sample were incubated at 5 °C for 10 min to quench the trypsin reaction.

Immunocapture

The sBCMA peptide enrichment was performed using the KingFisher Flex robot (Thermo Scientific, Waltham, United States) and Dynabeads M−280 beads (coated with streptavidin).

50 µL of the anti-peptide antibody solution at 80 µg/mL in PBS was added. The samples were agitated at slow speed for 12 h using the KingFisher Flex instrument. The beads were washed with PBS before being released into the samples. The beads were mixed for two hours at slow speed in the samples, allowing streptavidin–biotin coating, and then washed twice with PBS. The captured peptides were eluted in 100 µL of 1 % FA in three cycles of slow (5 min) and bottom mix (1 min) speeds.

LC MS/MS analysis

10 µL of each sample was injected on a UHPLC Nexera (Shimadzu, Kyoto, Japan) coupled to a SCIEX QTRAP 5500 (Sciex, Framingham, United States) instrument. Peptides were separated on a Biozen Peptide XB-C18 (2.1 × 100 mm),1.7 µm (Phenomenex, Torrance, United States) column at 50 °C. Mobile phase A (Water + 0.1 %FA) and B (ACN + 0.1 % FA) were operated with a gradient elution as follow: 0–2 min 2 %B, 2–10 min 2–25 %B, 10–11 min 25–95 %, 11–13 min 95 %B, 13–13.5 95–2 % B, 13.5–16 min 2 % B, with a flow rate of 0.25 mL/min. Retention times of the peptide and the internal standard were 6.2 min.

For the MRM analysis, the transition [m/z 710.9 $[M + 2H]^{2+} \rightarrow m/z$ 871.6 (y_7^+)] was the quantifier for the surrogate sBCMA peptide C(Cam) SSNTPPLTC(Cam)QR, and the transition [m/z 714.3 $[M + 2H]^{2+} \rightarrow m/z$ 878.4 (y_7^+)] was the quantifier for the internal standard C(Cam) SSNTPPL*TC(Cam)QR (L*: Leu [^{13}C6, ^{15}N]).

The ESI source was set at 600 °C, and the ion spay voltage was set at 4500 V. The nebulizing gas and desolvation gas were set at 55 and 45 psi. The curtain gas was at 20 psi. The collision energy was set at 34 V, the declustering potential at 90 V, the entrance potential at 10 V for the two transitions. The collision cell exit potential at 12 V for the light peptide transition and 15 V for the heavy peptide transition. The dwell time was set at 200 ms for both transitions.

Data were acquired and analyzed by the Analyst software from SCIEX (version 1.6.2). Calibration curves were constructed by plotting the corresponding peak area ratios of analyte/internal standard versus the corresponding analyte concentrations using weighted ($1/x^2$) regression analysis.

QCs and standards preparation

The dynamic range of the quantification method was 10–750 ng/mL. The calibrators were 10, 25, 50, 100, 250, 350, 500 and 700 ng/mL. Standards were prepared by spiking recombinant sBCMA into the surrogate matrix (monkey plasma). QC LLOQ was prepared by spiking 10 ng/mL of recombinant sBCMA into the surrogate matrix. QC LOW was constituted from a pool of human plasma (endogenous level calculated at 25 ng/mL). QC MID and QC HIGH were constituted from the same pool of human plasma and spiked at 300 and 600 ng/mL with recombinant sBCMA. The target determination of QC LOW, MID, and HIGH concentrations was performed at the beginning of the validation.

Parallelism test

Six individual batches of human plasma were used for the parallelism test. The endogenous level of sBCMA was determined in each lot (n = 6). One dilution (2-fold factor) in surrogate matrix was performed, and five concentrations of recombinant sBCMA over the range of the calibration curve were spiked into human plasma. All diluted and spiked samples were extracted (n = 3) and analyzed against a surrogate matrix calibration curve. Parallelism between the surrogate matrix and the sample matrix was demonstrated if, for at least four out of the six lots tested, the individual bias was less or equal than ± 20.0 % for 2/3 of the replicates at each concentration level.

Interference test

QC LOW and QC HIGH were spiked (n = 6) with two relevant concentrations of the monoclonal anti-BCMA Sanofi product (up to the expected pharmacokinetic concentrations) and two concentrations of potential interferent endogenous proteins (2 and 25 ng/mL of human BAFF protein and 2 and 100 ng/mL of APRIL). The spiked QC LOW/HIGH were incubated at 37 °C over four hours under 600 rpm agitation prior analysis to mimic the physiological process. The spiked QCs were analyzed against the nominal concentration determined for the non-spiked QCs. The test passed if the individual bias was less or equal than ± 20.0 % for at least 66 % of the replicates at each QC level.

Validation of the method

The validation was performed following in-house standard operating procedures based on the guidelines set forth in the M10 Bioanalytical Method Validation and Study Sample Analysis – Guidance for Industry, November 2022.

Accuracy and precision

Validation samples for sBCMA prepared at each concentration level (LLOQ, LOW, MID and HIGH) were analyzed (n = 6) against a surrogate matrix calibration curve, on four separate occasions.

Dilution linearity

The ability to quantify plasma concentrations of sBCMA at levels higher than the ULOQ 1000 ng/mL (ULOQ) was evaluated by diluting 10-fold a super HIGH QC (endogenous level in human plasma spiked with 5000 ng/mL) and 20-fold a very HIGH QC (endogenous level in human plasma spiked with 10,000 ng/mL) with surrogate matrix (n = 6). The super HIGH QC and very HIGH QC samples were analyzed against a surrogate matrix calibration curve, with run acceptance QCs.

Frozen stabilities

Validation samples at LOW and HIGH concentration levels (n = 6 per level) in human and monkey plasma were analyzed at t0 and after storage at −80 ± 10 °C for 1 and 3 months using freshly prepared calibration curves. Run acceptance QCs were added.

Results

Selection of surrogate sBCMA peptide

sBCMA was digested and analyzed by LC-MS/MS using an Orbitrap QExactive + instrument (Thermo Scientific, Waltham United States). MRM transitions and collision energies were optimized. Peptide sequences were searched using Basic Local Alignment Search Tool (BLAST). C(Cam)SSNTPPLTC(Cam)QR was the only unique peptide in the human proteome with acceptable ionization efficiency and MS2 fragmentation.

Choice of the surrogate matrix

Endogenous levels of sBCMA have been reported at approximately 20 ng/mL in healthy individuals [11]. To establish a calibration curve for the absolute quantification of sBCMA, a surrogate matrix and a recombinant protein were required.

Monkey plasma was assessed as a surrogate matrix for human plasma. The homologous sequence of the sBCMA peptide in cynomolgus monkeys is CSSTPPLTCQR, while the human sBCMA peptide (CSSNTPPLTCQR) is not found within the monkey proteome using BLAST.

During method development, a sample size of 50 µL was used, and a matrix effect was observed between human and monkey plasma. The IS peak area showed equivalent responses between the two species when the IS was added after the capture, but different responses were noted when the IS was added before the capture. These data suggested that the matrix effect occurred during the immunocapture step. To reduce the matrix effect, the sample size was reduced by half, and 25 µL of NH_4HCO_3 (50 mM) was added. The use of an internal standard undergoing the immunocapture step corrected any further variation between the surrogate matrix and human plasma.

To assess the surrogate matrix and the recombinant sBCMA a parallelism test was performed [20] (Fig. 1).

All levels for all batches were within the acceptance criteria. The monkey plasma was validated as the surrogate matrix.

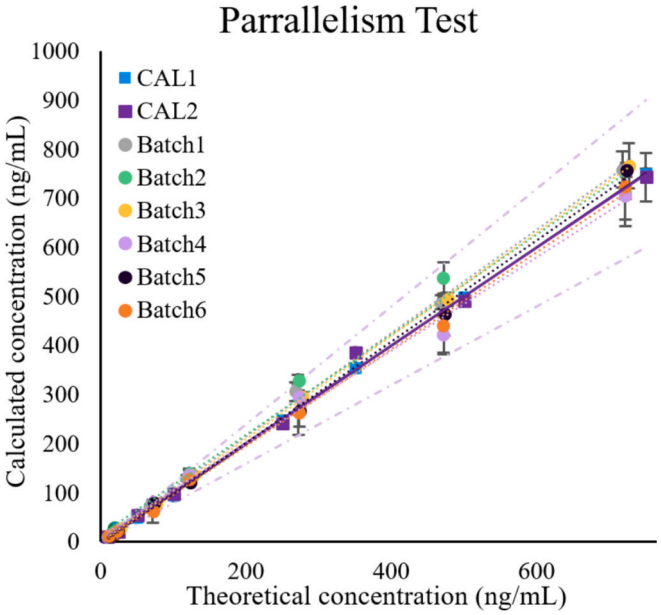

Fig. 1. Results of the parallelism test. CAL1,2: calibration curves in the surrogate matrix. Batch 1, 2, 3, 4, 5, 6: individual human plasma spiked at 50, 100, 250, 450, and 700 with recombinant sBCMA (n = 3) and diluted to half the original concentration in surrogate matrix (n = 3). Dash-dotted line: 20 % acceptance criteria.

Validation of the method

Representative chromatograms at LLOQ (in surrogate matrix) and endogenous level (authentic matrix) of the surrogate peptide are shown in Figs. 1 and 2 of the Supplementary Material.

Four runs of accuracy and precision were performed with the results provided in Table 1 of the Supplementary Material. The inter-run accuracies (and CVs) were −19.4 % (16.3 %) for QC LLOQ, 6.2 % (6.52 %) for QC LOW, −2.4 % (7.03 %) for QC MID, and −1.1 % (6.6 %) for QC HIGH. CVs were calculated with 24 replicates per level, at each level, and were within the acceptance criteria (20 % except for QC LLOQ at 25 %).

The matrix effect was evaluated in human plasma as part of the parallelism test. Dilution integrity in monkey plasma was evaluated with 10-fold and 20-fold dilution of human plasma spiked with 5000 and 10 ng/mL of sBCMA. The results are provided in Table 6 of the Supplementary Material and the %biases are within ± 10 %. The results of the frozen stability study in monkey and human plasma at −80 °C are provided in Table 5 of the Supplementary Material and show that there is no significant loss of signal at 1 and 3 months (bias <10 %).

All items of validation passed within the acceptance criteria (20 % except for 25 % at LLOQ). (Supplementary Material).

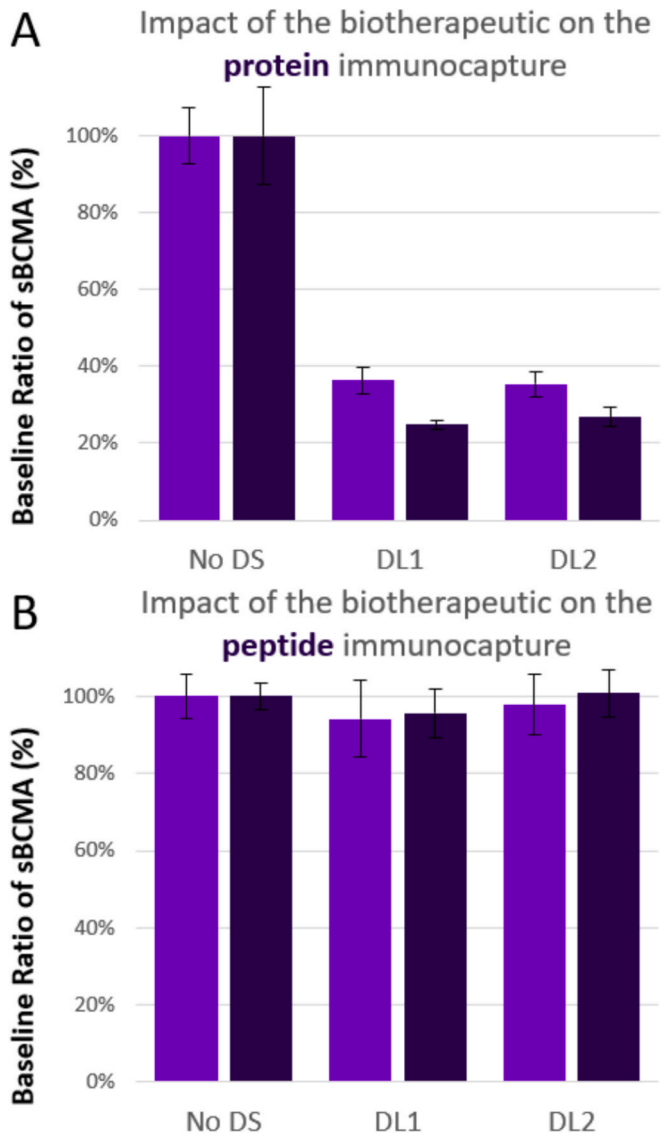

Fig. 2. Impact of the biotherapeutic on the protein (A) and peptide (B) immunocaptures. Purple (left): QC LOW (endogenous protein at ~25 ng/mL). Dark purple (right): QC HIGH (endogenous protein + recombinant protein spiked at 600 ng/mL). n = 6. Baseline ratio: concentration of the sample compared to the concentration without pre-marketed Sanofi product (No DS). Dose level 1 and 2 (DL1 and DL2) corresponds to the expected drug concentrations in patient samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Interference of biotherapeutics and endogenous proteins

The interference of the biotherapeutic in the protein level and the peptide level immunocapture was evaluated.

Fig. 2A shows a strong interference of the monoclonal anti-BCMA in the capture of sBCMA using the protein level immunocapture. A 60 % decrease in signal and sBCMA concentration was observed in samples in the presence of the drug at the expected concentration in the patient samples. Increasing concentrations of the capture antibody were tested but couldn't eliminate the interference. Depending on the affinity of the anti-BCMA therapy compared to the capture reagent, increased concentration of the anti-protein antibody might not overcome this issue.

Fig. 2B shows the elimination of the biotherapeutic interference in the peptide level immunocapture LC-MS/MS sBCMA analysis. All QCs were within the acceptance criteria of the validation. These results highlight the power of the peptide level immunocapture and the agility of mass spectrometry to overcome the weaknesses of protein-level immunocapture in hybrid and LBA assays.

In addition to the binding to the BCMA-targeting biotherapeutics, BCMA binds two ligands: A PRoliferation Inducing Ligand (APRIL) and B-cell activating factor (BAFF) [33]. The interference of these two endogenous proteins to the peptide-level immunocaptureLC-MS/MS approach was evaluated (Fig. 3) and all QCs were within the acceptance criteria (20 %). No endogenous protein interference was detected.

Overall, no interference was detected using the peptide level immunocapture LC-MS/MS analysis and the validation test was validated within the acceptance criteria (20 %).

Discussion

Anti-protein antibodies for sBCMA were commercially available, while the anti-peptide antibodies were custom-made for the analysis. The immunization of rabbits, purification, and biotinylation of the

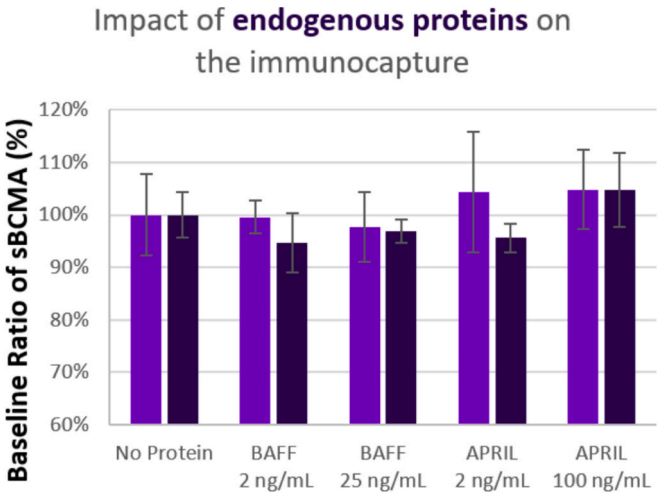

Fig. 3. Impact of the endogenous proteins on the peptide immunocapture. Purple (left): QC LOW (endogenous protein at 25 ng/mL). Dark purple (right): QC HIGH (endogenous protein + recombinant protein spiked at 600 ng/mL). n = 6. Baseline ratio: concentration of the sample compared to the concentration without endogenous protein (No Protein). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

polyclonal antibodies prolonged method development and increased the assay's cost. To circumvent the need for developing anti-peptide antibodies, the use of anti-protein antibodies for the affinity capture of tryptic peptides has been evaluated in a screening study [34]. However, depending on the epitope of the commercially available antibodies, the antibody may not target and therefore capture the peptide. Given the mass difference between sBCMA and the CSS peptide (~3.8), and the equivalent molar quantity of antibody required, the peptide immunocapture also consumed four times more capture reagent.

Hybrid assays involve multiple complex steps that can introduce variability due to one or two days of sample preparation. An internal standard is crucial for mitigating this variability. However, selecting the ideal internal standard can be challenging, especially when it needs to participate in the critical immunocapture step. With protein immunocapture, heavy proteins are usually expensive to produce and may not be commercially available. A flanked peptide is added after the immunocapture step in the protein-level immunocapture method. Peptide enrichment offers a more practical solution, utilizing heavy or flanked heavy peptides that are easier and more affordable to produce. The flanked peptide is added before the digestion step and undergoes the entire assay process, helping to minimize matrix effects and enhance the overall robustness of the method. This robustness is further improved through automation.

Current LBA methods and the protein immunocapture LC-MS/MS approach can experience drug interference when measuring total sBCMA. To address this issue, an acid or ionic dissociation step can be added; however, this may not be sufficient depending on the drug concentration. The strength of the peptide immunocapture LC-MS/MS approach lies in its ability to eliminate all interactions during the denaturation and digestion steps. Our study demonstrated the successful elimination of all drug and endogenous ligand interferences, enabling accurate quantitation of total sBCMA.

The LLOQ of the assay was adequate for sample analysis; however, other published anti-peptide immunocapture LC-MS/MS assays demonstrated superior sensitivity, achieving 0.1 ng/mL for serum thyroglobulin measurement [35]. To enhance the assay's sensitivity, micro-LC coupled with a more sensitive instrument, such as the SCIEX QTRAP 7500, could have been used.

The assay combines the sensitivity of LBAs with an immunocapture step that decomplexifies the matrix, along with the specificity of mass spectrometry through the detection of a specific surrogate peptide. Nonetheless, it also inherits the drawbacks of both methods, such as the need for specific critical reagents in immunoassays, the custom production of the anti-peptide antibody, and the low throughput of MS assays. Additionally, the extra sample preparation steps can increase the variability of the method.

Conclusion

The developed LC-MS/MS method using peptide immunocapture was fit-for-purpose validated based on the Context of Use for this biomarker, selecting the appropriate validation items. The method achieved a LLOQ of 10 ng/mL and eliminated interference from the monoclonal anti-BCMA product and endogenous proteins in the capture of sBCMA.

The peptide immunocapture method could potentially be adapted for the bioanalysis of anti-BCMA therapies that provoke a strong anti-drug antibody response, mitigate off-target interference, or accommodate drug substances that lose structural stability during sample storage. This represents a new perspective in mass spectrometry that could minimize the interference and challenges encountered in ligand binding and hybrid assays.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics statement

This study utilized only fully anonymized patient samples that were not obtained specifically for use in this study through an interaction or intervention with living individuals. Neither informed consent nor IRB review was required.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2025.04.006.

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