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METHOD

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Development and validation of an antidrug antibody assay for TRK-950 using Melon™ gel and SPEAD pretreatment

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ABSTRACT

Aim & methods: TRK-950, a humanized IgG1 monoclonal antibody against CAPRIN-1, which is reported to be expressed on the cell surface of various forms of cancer, is currently being developed for multiple types of solid cancer. We developed an antidrug antibody (ADA) assay that includes two pretreatment processes for use in clinical trials, namely, Melon™ gel treatment and solid-phase extraction with acid dissociation (SPEAD), to reduce baseline variability between individual serum samples and improve drug tolerance.

Results: Comparing the assay with or without the pretreatment process using commercially available human serum samples, the mean response calculated from 18 individual human samples decreased from 327 to 270, and the coefficient of variation decreased from 41% to 16%. The new assay with the two-step pretreatment met the criteria set by the regulatory agency throughout the validation study. We also confirmed the recovery rates of IgG, IgM, and ADA – drug immune complexes after treatment with Melon™ gel. Clinical trials (NCT05423262) employing this improved analytical method have been conducted, and the results confirm the low immunogenicity of TRK-950.

Conclusion: The combination of pretreatment with Melon™ gel and SPEAD is applicable for clinical ADA assays.

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KEYWORDS
Validation; sample preparation; method development; matrix effects; Immunoassay; biotherapeutics; assay

1. Introduction

TRK-950 is a recombinant, humanized monoclonal antibody that binds to CAPRIN-1 [1]. CAPRIN-1 has been reported to play a key role in the differentiation, proliferation, and malignancy of tumors [2,3]. TRK-950 exhibited therapeutic effects in a mouse tumor model and demonstrated extremely high tolerance in monkey toxicity studies, and it is currently under evaluation as a drug candidate in clinical trials targeting oncology indications. TRK-950 is being investigated as both a monotherapy and in combination with several approved therapeutics against advanced malignancies in Phase I, Ib and II clinical trials (NCT2990481/NCT03872947/NCT06038578/NCT05423262).

The administration of therapeutic biological drugs, including monoclonal antibodies, can induce immune responses in individuals [4–6]. The immune response leads to the formation of antidrug antibodies (ADAs). The generated ADAs could potentially affect the pharmacokinetics, pharmacodynamics, safety, and efficacy of the drug [7,8]. ADA assessment is a critical component in the development of therapeutic biological drugs, and well-designed and specific ADA immunoassays are crucial for appropriately monitoring the drug's immunogenicity profile. Immunogenicity assessment is required by regulatory agencies for the development and approval of biological drugs [9–11]. In oncology, an increasing number of targeted anticancer agents and immunotherapies are of biological origin. These biological drugs can trigger immune responses that lead to ADA formation. Research in this field is also accumulating knowledge about the effects of ADA formation [12].

To detect ADA in clinical settings, various guidelines and guidance documents have been issued by regulatory authorities [13,14]. Bridging immunoassay platforms, including enzyme-linked immunosorbent assay (ELISA) and electrochemiluminescence immunoassays, are often used to detect antibodies directed against therapeutic monoclonal antibodies. These analytical methods are designed to detect molecules with two binding sites for the drug, making it theoretically suitable for detecting immunoglobulins. Various experimental refinements, such as adding different pre-treatment steps [15–19], and improvements in statistical processing of the obtained results have been proposed [4]. Consequently, these methods have often been used as the standard approach to ADA analysis in biopharmaceutical development. Recent publications and regulatory agency guidance documents indicate the need for specific immunogenicity assays and a tiered testing scheme to support clinical immunogenicity investigations [4,5,11].

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Article highlights

- Immunogenicity assessment of biopharmaceuticals and evaluation of anti-drug antibody (ADA) development are critical for clinical trials. However, these analyses are influenced by a number of factors, including administered drugs and target molecules, necessitating methodological refinements to mitigate such interference (Introduction).
- TRK-950, a humanized monoclonal antibody targeting the cancer cell surface antigen CAPRIN-1, underwent clinical trials. Baseline variability of unknown origin was observed in its ADA analysis. To address this, a pretreatment method using Melon™ Gel, which selectively traps non-antibody molecules, was integrated into the ADA assay, followed by rigorous validation (Methods).
- Pretreatment with Melon™ Gel reduced variability in measurements of naïve human serum samples. (Results: Analysis of the purification characteristics of Melon™ gel).
- Human IgG was not trapped by Melon™ Gel, while the majority of IgM was partially retained.
- ADA-TRK-950 complexes were not trapped by Melon™ Gel (Results: Analysis of the purification characteristics of Melon™ gel).
- Since Melon™ Gel elutes ADA-drug complexes without dissociating them, combining it with acid-dissociation (SPEAD) pretreatment enhanced drug tolerance for high-sensitivity ADA detection. (Results: Improvement of drug tolerance by SPEAD pretreatment).
- The developed ADA assay met regulatory guidelines in validation study, demonstrating robust analytical performance and reliability. (Results: Validation of ADA assay with Melon™ gel and SPEAD pretreatment).
- The pretreatments reduced baseline variability, enabling higher ADA detection performance. Clinical analyses with this ADA assay, though based on a limited sample size, demonstrated the expected characteristics, suggesting its reliability in assessing the immunogenicity of TRK-950, which is currently under clinical development. (Discussion).
- The molecular origin of variability remains unidentified but is unlikely to involve the drug's target protein. While contributing to assay variability, its estimated concentration is extremely low, posing minimal risk of impacting pharmacokinetics/pharmacodynamics. (Discussion).

ADA detection methods can be susceptible to interfering substances present in the test matrix. The most important interfering substance in an ADA detection assay is the drug itself, which can lead to false-negative assay results. To mitigate this interference, various pretreatment methods have been proposed [15–19]. Examples include the acid dissociation method, which dissociates the drug and ADA in the serum sample, and more efficient methods such as SPEAD (solid-phase extraction with acid dissociation) and BEAD (bead-extraction and acid dissociation), which use the drug as a tool molecule to capture ADA by affinity and purify the sample using solid-phase surfaces or beads. Additionally, the PandA (precipitation and acid dissociation) method, which uses excess drug and PEG molecules for purification, has been proposed [20]. Furthermore, methods to increase the specificity of ADA detection, immune complex ADA assay using FcR receptor proteins or modified drugs, have also been reported [21].

It is underappreciated that the drug's target can also be an interferent depending on the drug/target molecules. For monoclonal antibodies, in particular, the drug can form extended complexes with soluble target ligands (including soluble forms of some cell-surface receptors, also called shedding proteins) and remain in circulation for long periods [15,21–24].

In most cases, the amount of the drug target in assay samples varies with the disease state, treatment regimens and schedules, or regulation of endogenous proteins, which can influence the measurement of ADAs. Additionally, other molecules with the same structure or affinity for therapeutics could also influence the ADA assay results. These pretreatment methods are also considered effective ways of removing these molecules. The concentrations of these interfering molecules are not usually monitored in clinical trials even though they could interfere with ADA measurement. This could result in either false-positive or false-negative results depending on the concentration, structure, and chemical and biological properties of the molecules.

Given that the purpose of the ADA assay is immunogenicity assessment, the assay target should be confined to endogenous antibodies (immunoglobulin molecules). It is preferable to remove non-immunoglobulin molecules, including the target protein, which interfere with the assay. The methods for eliminating these molecules need to be evaluated for each drug target, and a standardized approach has not been established in the industry. When interfering molecules are clearly identified, studies have reported purification strategies such as the removal of interfering molecules using affinity purification. Additionally, methods to eliminate non-antibody molecules in samples have been explored, including purification with Protein A, which has high affinity for most immunoglobulins, and Melon™ Gel, which selectively binds to non-antibody molecules [25,26].

In this study, we developed an ADA analysis method for clinical trials of TRK-950. The method involves a two-step pretreatment process using Melon™ gel and solid-phase extraction with acid dissociation (SPEAD). This method aims to address baseline variability and improve drug tolerance. Furthermore, we thoroughly examined the effects of these pretreatments on the assay.

2. Materials and methods

2.1. Equipment

The Spectra Max M5 plate reader (Molecular Devices Corp.) was used to measure the IgG/IgM concentrations. SECTOR S600 (Meso Scale Diagnostics, LLC) was used for signal detection of the MSD plates in the ADA assay. MULTI-ARRAY 96-well Streptavidin Gold plates for the SECTOR Imager (Cat. No. L15SA, Meso Scale Diagnostics, LLC) were used for the ADA assay. A 96-well streptavidin-coated plate (Cat. No. 15500, Thermo Fisher Scientific Inc.) was used for the SPEAD pretreatment.

2.2. Standard ADA and reagent

TRK-950, a humanized anti-CAPRIN-1 antibody, was developed from a rabbit monoclonal antibody at Epitomics, Inc [1]. The reference ADA against TRK-950, Anti-id#126, a rabbit monoclonal antibody, was developed at Abcam by immunizing the Fab fragment of TRK-950. The Melon™ gel Spin Plate Kit for IgG Screening (Melon™ gel 96-well spin plate, Melon™ gel purification buffer, wash plate, and collection plates, Cat No. 45208, Thermo Fisher Scientific Inc.) was used to remove non-immunoglobulin proteins from serum. Biotin-labeled TRK-

950 was developed using EZ-Link Sulfo-NHS-LC-LC-Biotin (Cat No. 21338, Thermo Fisher Scientific Inc.). SULFO-Tag-labeled TRK-950 was developed using SULFO-Tag NHS-Ester (2 µmol, Cat No. R91AO–2, Meso Scale Diagnostics, LLC). MSD Read Buffer T (4×) with Surfactant (Cat No. R92TC, Meso Scale Diagnostics, LLC) was used to read the instrument responses. Individual human serum samples were purchased from BioIVT, Inc. A human IgG ELISA kit (E88–104, Bethyl Laboratories, Inc.) and human IgM ELISA kit (E88–100, Bethyl laboratories, Inc.) were used to measure human IgG/IgM concentrations.

2.3. Floating bridging LBA for the TRK-950 ADA assay

ADAs (anti-TRK-950 antibodies) in human serum were measured using a sandwich LBA assay employing a floating and bridging method. Briefly, samples (including positive and negative controls) were mixed with an equal volume of a labeled drug mixture (1.125 µg/mL of biotin-labeled TRK-950 and 1.125 µg/mL of SULFO-labeled TRK-950 in 0.475% Block Ace) and incubated for 90 minutes. Fifty µL of the samples was added to avidin-coated micro plates blocked with 1% Block Ace and incubated for 60 minutes. The microplate was washed three times, MSD Read Buffer was added, and then the microplate was incubated for 1 minute and ECL signals were measured.

2.4. Floating bridging LBA for detecting ADAs against TRK-950 in clinical trials

In clinical trials, anti-TRK-950 antibodies (ADAs) in human samples were assessed using the regulatory agency-recommended three-tiered approach [14]: screening assay, confirmatory assay, and titration assay. In the screening assay, the ADA levels in human serum samples were measured, and samples exceeding a predefined cut point (set to yield a 5% false-positive rate) were considered positive. Positive samples were further evaluated in the confirmatory assay. In this assay, excess drug (TRK-950) was added to samples to achieve a final concentration of 10 µg/mL, a confirmatory ADA assay was then conducted, and samples that exhibited inhibition exceeding a predefined cut point (statistically determined beforehand to have a 1% false-positive rate) were considered positive, indicating the presence of drug-specific ADAs. ADA levels in confirmatory assay-positive samples were then semiquantified by serial dilution and measurement of the sample.

2.5. Pretreatment with Melon™ gel

In the method for removing non-immunoglobulin molecules from human serum using Melon™ gel, human serum samples were diluted 1.5-fold with Melon™ gel purification buffer, 50 µL of diluted samples was added to the Melon™ gel spin plate, and mixed with the gel. After incubation at room temperature for 5 minutes, the plate was centrifuged at 1000 g for 1 minute and the eluate was collected. Melon™ gel purification buffer (50 µL) was added and incubated for 5 minutes, the plate was centrifuged at 1000 g for 1 minute and the eluate was collected (total: 100 µL) as the Melon™ gel treated sample.

2.6. SPEAD pretreatment

To extract ADAs from serum samples, after obtaining samples following Melon™ gel pretreatment, samples were diluted 10-fold with 1% Block Ace, and an equal volume of 600 mmol/L acetic acid was added. The mixture was incubated at room temperature for 30 minutes with shaking. Biotin-labeled-TRK-950 (30 µL, 21.7 µg/mL) was added to this solution (100 µL), which was incubated at room temperature for 90 minutes. The mixture (100 µL) was added to streptavidin-coated plates blocked with 1% Block Ace and incubated at room temperature for 30 minutes, and washed three times. Three hundred mmol/L of acetic acid (80 µL) was added to the plates and incubated for 5 minutes to recover the purified ADAs. Samples were immediately neutralized by adding an equal volume of labeled drug mixture prepared based on 0.3 mol/L of Tris HCL (pH 9.5).

2.7. Detection of IgG/IgM

The concentrations of IgG and IgM were measured in accordance with the instructions provided with the detection kits. Briefly, standard solutions and assay samples (100 µL) were added to the assay plate and incubated for 60 minutes. The microplate was washed four times. Human IgG/IgM detection antibody (100 µL) was added to the plate, and incubated for 60 minutes and washed four times. HRP-labeled detection antibody solution (100 µL) was added and incubated for 30 minutes, and the plate was washed four times. TMB substrate was added, and the plate was incubated for 30 minutes. Stop Solution was added, and the absorbance was measured at 450 nm.

2.8. Data analysis

Data analysis was conducted using Microsoft Excel and Discovery Workbench Version 4.0. A response curve was constructed by importing the ECL responses into SoftMax Pro GxP Version 7.0.3.

2.9. Validation study for anti-TRK-950 ADA assay in clinical trials

A validation study for the method of detecting anti-TRK-950 antibodies was conducted in accordance with the FDA guidance [14]. For the calculation of the cut point, we used serum samples from 52 individuals (26 males comprising 17 Caucasians, 4 hispanics, and 5 Africans; and 26 females comprising 17 Caucasians, 4 hispanics, and 5 Africans) and performed distribution assessment using box plots, iteratively removing outlier individuals until no outliers remained in the box plot analysis to choose serum samples for cut point determination. The screening cut point, confirmatory cut point, selectivity, intra-assay precision, inter-assay precision, selectivity, hook effect, drug tolerance, concomitant drug effect, titration assay precision, freeze – thaw stability, bench-

top stability, and short-term stability were determined. The conditions are presented in detail in Table 1.

2.10. Clinical trial of TRK-950 and ADA analysis

A Phase I clinical trial of TRK-950 was conducted in patients with advanced solid tumors (NCT05423262). TRK-950 was administered once weekly as a monotherapy (5 or 10 mg/kg) or in combination with nivolumab. ADA levels were measured monthly prior to TRK-950 administration. Serum samples with drug concentrations below the drug tolerance of the ADA assay were evaluated, and their measurements in the screening assay were expressed as a normalized response ratio relative to negative samples.

3. Results

3.1. Development of LBA assay for detection of ADA with Melon™ gel and SPEAD pretreatment

Melon™ gel binds nonantibody serum proteins such as albumin and transferrin at physiological pH, thereby fractioning and recovering immunoglobulin from the flow-through in a mild buffer, making it suitable for downstream applications [26,27]. We purified anti-TRK-950 antibodies from human serum using Melon™ gel and SPEAD treatment to remove nonantibody proteins and residual drugs that can cause response variance in the ADA assay (Figure 1).

The anti-drug antibody (ADA) quantification method was established as a bridging ligand-binding assay (LBA) format

Table 1. Validation results of the newly developed analytical method and conventional acid management method.

	Condition	Result
Cut point	samples: 52 individuals of healthy donors, 6 different assays (2 analysis) 26 males: 17 of Caucasian, 4 of Hispanic, 5 of African 26 females: 17 of Caucasian, 4 of Hispanic, 5 of African	Screening cut point: 1.07 Confirmatory cut point: %Depletion: 18.1
Sensitivity	samples: 3.00, 10.0, 30.0, 100, 300, and 1000 ng/mL ($n = 1$ each, 6 batches)	6.80 ng/mL
Selectivity	samples: 10 individuals of healthy donors (5 males and 5 females) , hemolyzed serum (1 individual), and hyperlipidemic serum (1 individual)	Accepted
Drug tolerance	samples: 100 and 10,000 ng/mL Drug: 0, 100, 300, and 600 µg/mL ($n = 1$ each, 1 batch)	Positive control: drug tolerance limit 100 ng/mL: 600 µg/mL 10000 ng/mL: 600 µg/mL
Intra-assay precision	samples: 0, 16.4, 1000, and 10,000 ng/mL ($n = 6$ each, 1 batch)	Screening assay: %CV: 2.8% to 6.9% Confirmatory assay: %CV: 0.1% to 6.5%
Inter-assay precision	samples: 0, 16.4, 1000, and 10,000 ng/mL ($n = 3$ each, 6 batches)	Screening assay: %CV: 2.7% to 19.8% Confirmatory assay: %CV: 0.1% to 16.8%
Hook effect	samples: 5000, 10000, 20000, 40000, 80000, and 100,000 ng/mL ($n = 3$ each, 1 batch)	No hook effect: up to 100,000 ng/mL
Freeze-thaw stability	samples: 16.4 and 10,000 ng/mL ($n = 3$ each) Frozen (−70°C or below) and thawed (at room temperature), 3 and 5 cycles	5 cycles
Bench-top stability	samples: 16.4 and 10,000 ng/mL ($n = 3$ each) Room temperature, 24 hours	Accepted
Short-term stability	samples: 16.4 and 10,000 ng/mL ($n = 3$ each) 2°C to 8°C, 24 hours	Accepted

A validation study for the method of detecting anti-TRK-950 antibodies was conducted in accordance with the FDA guidance [14]. After the calculation of the cut point, the validity of the analytical method was confirmed in accordance with the items listed in the table during the GLP-compliant validation study.

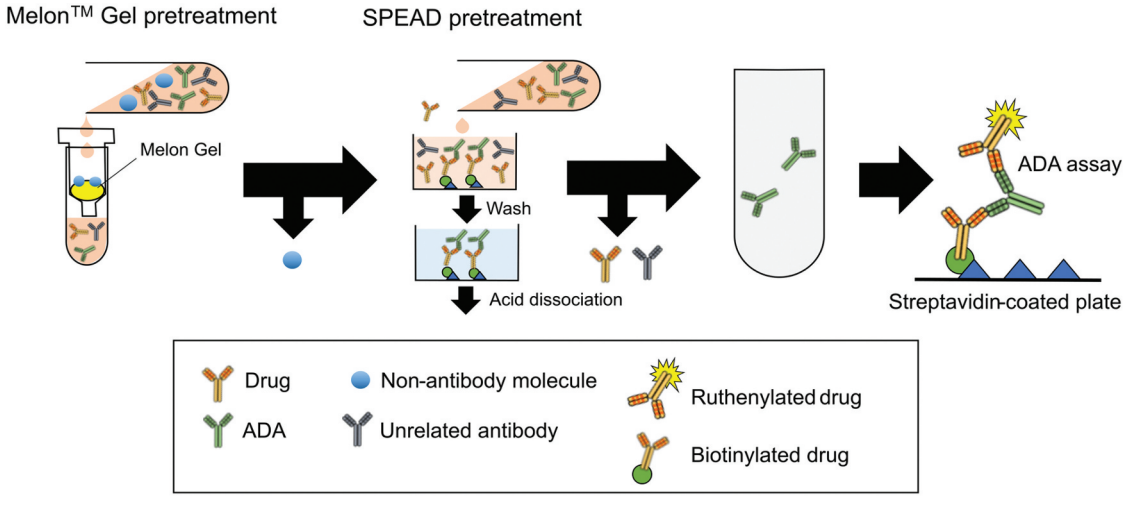

Figure 1. Schematic representation of a new assay for detecting anti-TRK-950 antibodies in human serum. As a preliminary step to the commonly performed floating bridging LBA assay, sample pretreatment was conducted using Melon™ gel and the SPEAD method. With Melon™ gel, non-antibody molecules are removed and the antibody fraction is recovered. With SPEAD, only antibodies with affinity for the drug are purified from the antibody fraction, while other antibody molecules, including free drug, are removed.

using Biotin-TRK-950 as the capture antibody, SULFO-TRK-950 as the detection molecule, and MSD-ECL (electrochemiluminescence). Antibody concentrations were optimized through checkerboard titration to achieve maximum sensitivity and a linear dynamic range. Ultimately, the concentration of both antibodies became 0.5625 µg/mL in the sample-antibody mixture after optimization. This protocol balanced signal-to-noise ratio with dose-responsive proportionality across the assay's working range (Supplementary Figure S1).

3.2. Analysis of the purification characteristics of Melon™ gel

The responses and variability of the ADA assay using 18 individual serum samples with and without Melon™ gel treatment are presented in Figure 2 as ECL signals and as ratios relative to the pooled serum used as a negative assay control (set to 1.0). Prior to Melon™ gel treatment, there was significant inter-individual variability, with some serum samples showing high values. After Melon™ gel treatment, the number of samples exhibiting high values decreased, and the overall variability was reduced. The mean ECL signal decreased slightly from 327 to 270 upon Melon™ gel treatment, from 1.26 to 1.11 as ratios, whereas the coefficient of variance (CV) of the responses as ECL signals decreased substantially from 41% to 16%.

According to the FDA guidance for immunogenicity testing, the initial screening assay should be able to detect all relevant immunoglobulin isotypes [14]. Considering that TRK-950 is administered intravenously via nonmucosal routes, for which the risk of anaphylaxis is considered to be lower, the relevant ADA isotypes that need to be analyzed in the ADA assay are IgM and IgG. We measured the concentrations of IgG and IgM in serum samples from 10 healthy volunteers and 10 patients with cancer before and after Melon™ Gel treatment and calculated the recovery rates. (Figure 3(a,b)). The results indicated that the IgG recovery rate was 100.3% ± 16.4% in healthy individuals, versus 95.7% ± 4.7% in patients with cancer (Figure 3(a)). The IgM recovery rate was 23.8% ± 9.2% in healthy individuals, compared with 28.3% ± 4.7% in patients with cancer (Figure 3(b)). Thus, there was no difference in recovery between patients and healthy individuals.

ADA could be present in blood in two forms, namely, as a free antibody or as part of an ADA – drug immune complex. To confirm the recovery of ADA – drug immune complexes by Melon™ gel treatment, the standard ADAs with various drug concentrations were added to serum samples prepared from healthy donors or patients with cancer to form ADA – drug immune complexes, and the recovery rates from Melon™ gel were assessed by the ADA assay with SPEAD pretreatment (Figure 3(c,d)).

The recovery rate of ADA was calculated and compared as the signal ratios of the samples by comparatively measuring the solutions before and after Melon™ gel treatment. In the healthy human serum without drug, the ADA recovery rates were 80.5% for 0.1 µg/mL ADA and 70.9% for 10 µg/mL ADA. In the healthy human serum with drug, the ADA recovery rates ranged from 87.0% to 113.1% at 0.1 µg/mL ADA and from 76.6% to 110.8% at 10 µg/mL ADA. In the serum from cancer patients without drug, the ADA recovery rates were 98.5% at 0.1 µg/mL ADA and 75.8% at 10 µg/mL ADA. In serum from cancer patients with drug, the ADA recovery rates ranged from 88.4% to 115.7% at 0.1 µg/mL ADA and from 79.7% to 101.6% at 10 µg/mL ADA. These results illustrated that the ADA recovery rate after Melon™ gel treatment was not significantly affected by the existence of drug.

3.3. Improvement of drug tolerance by SPEAD pretreatment

We confirmed that the ADA-drug complexes were recovered from Melon™ gel pretreatment by ADA assay with SPEAD pretreatment. Subsequently, we assessed the necessity of implementing SPEAD treatment after Melon™ gel pretreatment (Figure 4). In the detection of serum with 50 ng/mL standard ADA, in the absence of SPEAD treatment, ADAs were detectable in the presence of the drug at 100 µg/mL, but in the presence of the drug at 200 µg/mL, the normalized response ratio (instrument response with ADA/instrument response without ADA) approached 1, making detection

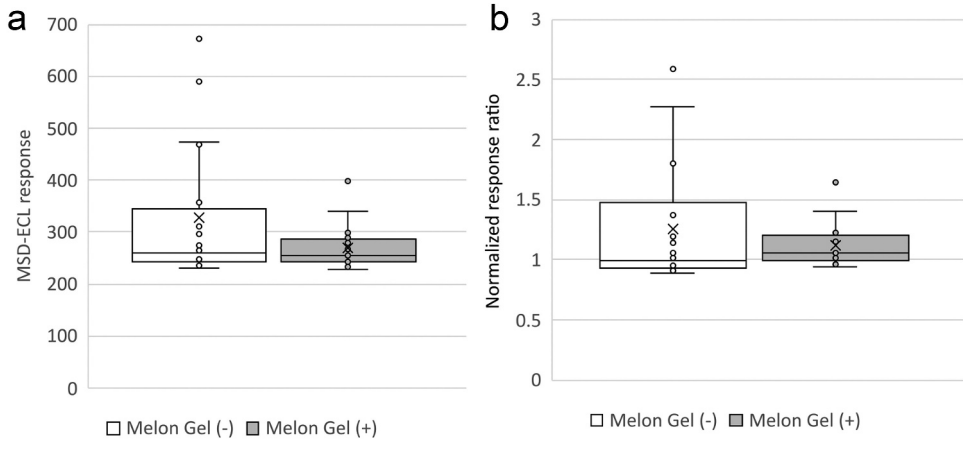

Figure 2. Comparison of the response and variation in ADA analysis with and without Melon™ gel.

Serum samples from 18 healthy individuals were analyzed in the ADA assay with or without Melon™ gel pretreatment. The individual values and the variation are represented by box plots as MSD-ECL responses (a) and the normalized response ratio (b).

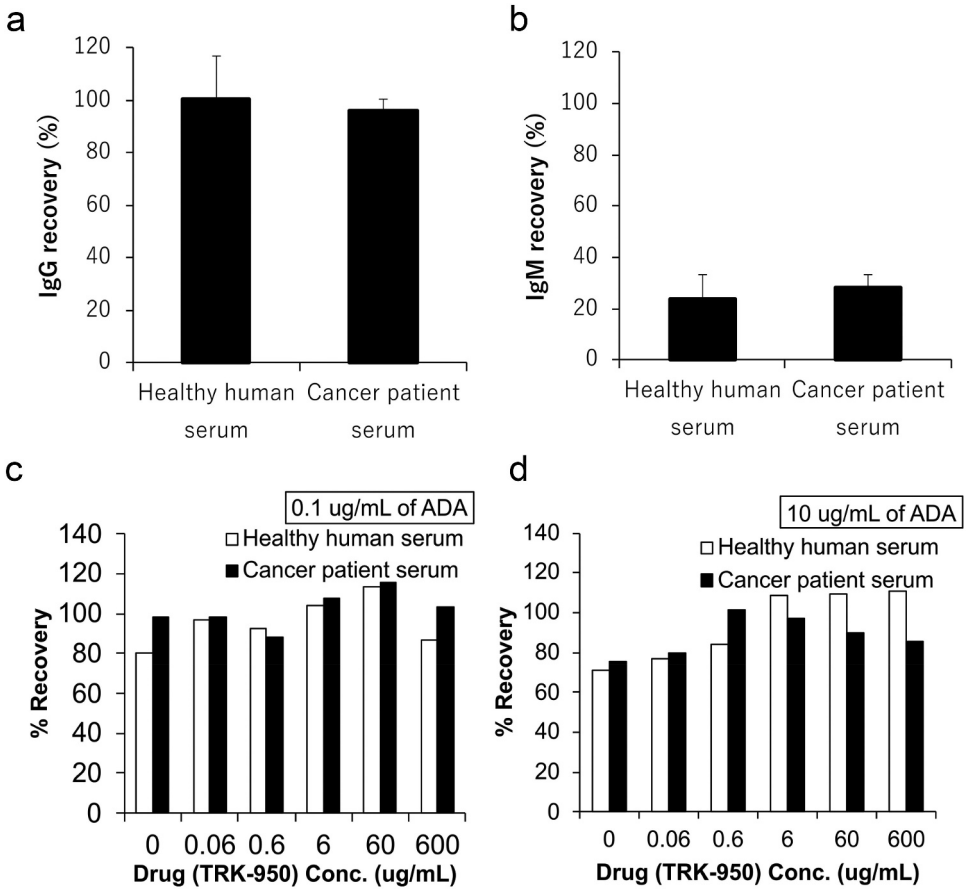

Figure 3. Analysis of the purification characteristics of Melon™ gel.

Recovery of endogenous IgG and IgM in serum samples from Melon™ gel treatment (a, b). The concentrations of IgG and IgM in naïve serum from healthy individuals ($n = 10$) and patients with cancer ($n = 10$) were measured using an IgG/IgM detection kit before and after Melon™ gel treatment. The difference in concentration is described as the recovery rate (% ± SD, concentration after Melon™ gel treatment/concentration before Melon™ gel treatment). Recovery of ADAs in the presence of drugs after Melon™ gel treatment (c, d). Pooled human serum from 56 healthy individuals (28 males and 28 females) and pooled serum from 15 patients with cancer (gastric cancer, $n = 5$; kidney cancer, $n = 5$; ovarian cancer, $n = 5$) were spiked with ADAs (A: 0.1 µg/mL, B: 10 µg/mL) and several concentrations of TRK-950. These samples were analyzed by the ADA assay with Melon™ gel pretreatment and SPEAD pretreatment. The difference in response was compared and defined as the recovery rate (%).

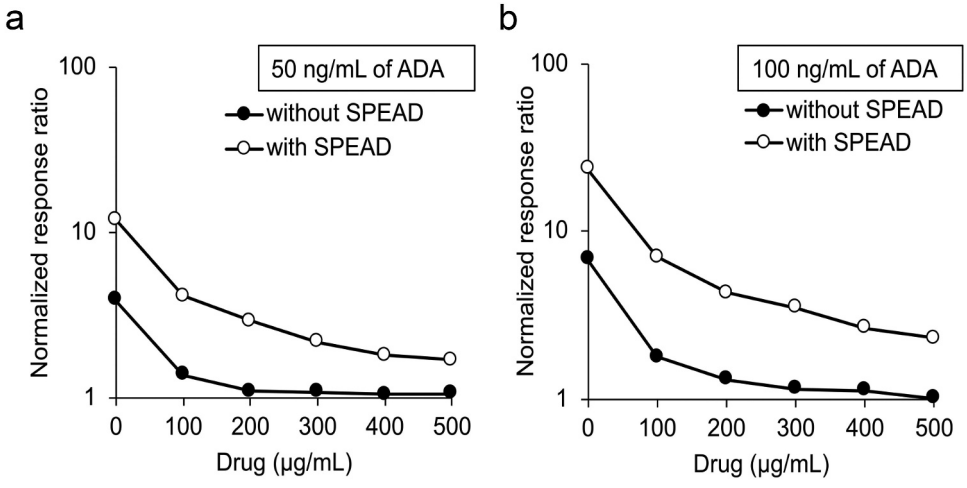

Figure 4. Improvement of drug tolerance by SPEAD pretreatment.

The pooled healthy serum samples were spiked with ADA [50 ng/mL (a), 100 ng/mL (b)] and several concentrations of TRK-950. These samples were initially pretreated with Melon™ gel and also with or without SPEAD method. The samples were analyzed by the ADA assay. The response is represented as the normalized response ratio of MSD-ECL responses in comparison to pooled unspiked serum from healthy individuals.

impossible. With SPEAD treatment, a ratio of approximately 2 was maintained up to drug concentrations of 500 µg/mL (Figure 4(a)). To detect 100 ng/mL standard ADA, the ratio approached 1 at a drug concentration of 300 µg/mL in the absence of SPEAD treatment, making detection difficult. However, with SPEAD treatment, the ratio was kept to approximately 3 even at drug concentrations of 500 µg/mL (Figure 4(b)). These data show that pretreatment with Melon™ Gel alone resulted in decreased sensitivity in the presence of the drug. However, when combined with SPEAD treatment, both sensitivity and drug tolerance were improved. This combination approach was confirmed to enhance the assay's performance in drug-containing samples.

3.4. Validation of ADA assay with Melon™ gel and SPEAD pretreatment

For the ADA analysis method in clinical trials for TRK-950, a two-step sample pretreatment process that included the removal of nonantibody molecules using Melon™ gel and SPEAD treatment, was applied to the ADA assay, and a validation study was conducted to confirm the performance and robustness per FDA guidance [14]. The results of the validation study are presented in Table 1. The assay consists of three tiers: screening, confirmatory, and titration. For the calculation of the cut point, we used serum samples from 52 individuals. We performed distribution assessment using box plots, iteratively removing outlier individuals until no outliers remained in the box plot analysis. The cut point for the screening assay was determined to be 1.07 as a normalized ratio set to achieve a 5% false-positive rate in accordance with the guidance. In the confirmatory assay, the inhibition rate upon addition of a sufficient amount of the drug was evaluated, and the cut point was determined to be 18.1% as a depletion set to achieve a 1% false-positive rate, in accordance with the guidance. For the titration, the samples post-Melon™ gel treatment were serially diluted, and the titer was assessed semi-quantitatively.

The ADA-response curve demonstrated excellent linearity across the concentration range of 3–1000 ng/mL (Supplementary Figure S1), with a sensitivity of 6.8 ng/mL established as the cut point-equivalent ADA reference concentration. Low (16.4 ng/mL) and high (10,000 ng/mL) quality controls (LQC/HQC) showed no interference in selectivity assessments, including analyses with hemolyzed and hyperlipidemic individual-specific serum samples. The validated drug tolerance (600 µg/mL) exceeds clinically anticipated blood drug concentrations, ensuring reliable ADA detection in therapeutic settings. Linearity confirmation up to 100,000 ng/mL precludes hook effect-related inaccuracies, consistent with FDA-recommended sensitivity thresholds for ADA assays.

3.5. ADA analysis in a clinical trial

The results of the ADA assay conducted using the established analysis as part of clinical trials are presented in Figure 5. The mean normalized response ratio of predose serum samples from 13 patients (13 samples) was 1.013 ± 0.107, and the mean value for postdose samples (50 samples) was 1.035 ± 0.0431. Based on the screening cut point (1.07) and confirmatory cut point (18.1%) determined using healthy human serum in the validation study, the predose positive rate (false-positive) was 7.6% (1/13) in both the screening and confirmatory assays. The postdose positive rate in the screening assay was 28% (14/50), and the rate in the confirmatory assay was 4% (2/50). The samples that tested positive in the confirmatory assay,

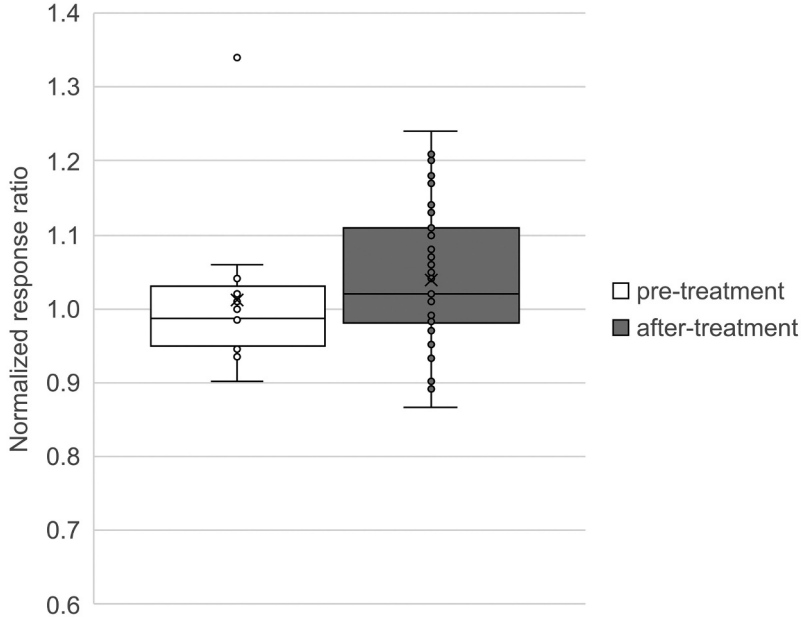

Figure 5. ADA analysis in a clinical trial.

The established and validated ADA assay was used in a clinical trial of patients with advanced solid tumors (NCT05423262). In the clinical study, TRK-950 was administered once weekly as a monotherapy (5 or 10 mg/kg) or in combination with nivolumab. Serum samples for ADA analysis were collected monthly prior to TRK-950 administration (a week after the previous administration of TRK-950). The serum samples with drug concentrations below the drug tolerance of the ADA assay were evaluated by the ADA assay including Melon™ gel and SPEAD pretreatment. The results of the ADA assay are represented by box plots as the normalized response ratio (13 patients, 13 predose samples, 50 postdose samples).

including pre- and post-dose samples, were from the same patient, indicating that no ADA formed because of TRK-950 administration.

4. Discussion

In this study, we developed a new ADA assay with Melon™ gel and SPEAD pretreatment to reduce the levels of nonantibody proteins in samples that cause interindividual differences and drug interference. In addition, we applied the new ADA assay to clinical sample analysis to evaluate immunogenicity.

When commercially available human serum was subjected to Melon™ gel treatment and SPEAD treatment, the response did not change significantly, whereas the variability was decreased (Figure 2). The same result is obtained with pretreatment with Protein A/Protein L, which also purifies immunoglobulin (data not shown), and these results indicate that the source of the variation in individual human serum samples is occurring in nonantibody molecules.

In the ADA assay format applied in this study, namely, the floating bridging LBA assay, the molecules that can theoretically lead to false-positive results are those with two binding sites for the target protein of the drug. Antibody and nonantibody molecules are considered potential candidates for these molecules.

A representative candidate antibody for false-positive molecules is rheumatoid factor. This is an antibody against the Fc region of human IgG, and it is known that patient serum containing rheumatoid factor exhibits elevated responses. Although the presence of rheumatoid factor is unrelated to the administered drug, following the intent of regulatory guidance, it might be desirable to perform the measure without removing this factor. Additionally, the existence of soluble Fc receptor proteins has been reported [21]. These proteins can bind to immunoglobulins including drug – antibody complexes and ADAs. However, it is unlikely that they would affect the ADA analysis because of their concentrations and other factors. However, when nonantibody molecules are the cause of false-positive results, it is considered appropriate to eliminate their influence prior to analysis.

In the established ADA assay including Melon™ gel pretreatment, the mean ECL response and CV decreased, as did the positive response in naïve human serum. These results suggest that nonantibody molecules interfered with the ADA assay.

It became evident that nonantibody molecules in serum can influence ADA measurements. In such cases, the first candidate for interfering molecules is typically the target molecule of the drug. Even membrane proteins that are not normally present in the blood can become soluble under certain conditions, leading to increased blood concentrations [15,28]. These molecules are called shedding proteins, and it is known that such molecules can affect the analysis and drug efficacy in pharmaceutical development. In addition, the existence of soluble Fc protein was reported in some patients under certain clinical conditions [29].

In this instance, CAPRIN-1 is generally the first candidate considered as an interfering molecule. Although TRK-950 has only one binding site in the CAPRIN-1 molecule, CAPRIN-1 has been reported to form multimers [2,30]. If multimeric forms of CAPRIN-1 are present in serum samples, they can potentially interfere with the measurement.

Nonetheless, when evaluating human serum that displayed high responses in the ADA assay using multiple types of anti-CAPRIN-1 antibodies, we were unable to confirm the existence of CAPRIN-1 protein (data not shown). This result indicates that CAPRIN-1 is not the major interfering molecule in the ADA assay. Additionally, attempts to identify the causative molecule using several different methods, including immunoprecipitation with various antibodies and proteomic approaches, have not yet led to its identification, and this remains a challenge for future research. The method of removing all nonantibody molecules using Melon™ gel might be useful for ADA assay in clinical development, as it can eliminate measurement interference even in cases in which molecular identification is difficult.

The inability to concentrate and detect the interfering molecule through immunoprecipitation and other methods suggests that the causative molecule has extremely weak affinity for TRK-950 or low binding specificity. Furthermore, based on the responses detected in the ADA assay, the calculated concentration of the observed interfering molecule was extremely low (<1 μg/mL). Considering the blood concentration of TRK-950 in clinical samples (>10 μg/mL), the interfering molecule has little to no impact on clinical efficacy.

We initially considered using Protein A/L and confirmed that the method works in a manner similar to the Melon™ gel method. When individual human serum samples were separated into antibody and non-antibody fractions using Protein A/L and each fraction was measured in an ADA assay, many of the serum samples that showed high responses before fractionation exhibited high responses in the non-antibody fraction (Protein A/L non-binding components) (data not shown). However, the necessary elution and purification processes were complex, and the recovery rates in spiking experiments were low, making it difficult to obtain consistent results. There were concerns about decreased analytical precision when applying this method to clinical analysis. Additionally, this pretreatment process depends on the affinity for the drug, and there is a concern that low-affinity ADAs should be removed from the samples. However, Melon™ gel has a long history of being used in research to remove nonantibody molecules, and it was previously assessed as a pretreatment method in clinical bioanalysis [26]. Unlike Protein A/L, Melon™ gel can remove nonantibody molecules in a mild solution without elution, permitting the isolation of the target antibody molecules [27]. This approach also avoids unnecessary dilutions that could reduce the sensitivity of the assay.

The recovery of IgM after Melon™ gel purification was 23.8% in normal human serum and 28.3% in serum from patients with cancer (Figure 3), indicating that the incidence of IgM-type ADAs might be underestimated. However, the value of IgM detection in the ADA assay in clinical trials is currently under discussion. In general, samples collected up to 14 days after the first dose are suitable for early IgM response assessment [31]. In the clinical trial

of TRK-950, ADA evaluation started in week 3 after the initial dose, and it was conducted continuously at approximately monthly intervals for each treatment cycle. Therefore, it was considered more important to have an evaluation technique that detected IgG more accurately than to detect IgM.

There was concern about whether the antibody – target complexes present in the serum samples would elute properly upon Melon™ gel treatment. However, it was confirmed that they eluted without issues (Figure 4). It was confirmed that this characteristic was consistent regardless of whether the serum was from healthy human serum and serum from patients with cancer. Although the separation mechanism of Melon™ gel is undisclosed, it is considered that the complex also has characteristics suitable for elution with Melon™ gel.

Ultimately, we developed an ADA analysis method by combining Melon™ gel and SPEAD pretreatment. Although this two-step pretreatment caused some sample loss and additional dilution, we were able to validate that the ADA assay complies with FDA guidance [14]. In the clinical trial using this ADA analysis method, although one patient tested positive in the confirmatory assay before and after drug administration (1/13), no treatment-induced ADAs were detected, confirming the low immunogenicity of TRK-950. Although the positive rate in the screening assay remained relatively high [pre-treatment: 7.6% (1/13), after treatment: 28% (14/50)], all samples tested negative in the confirmatory assay. This was likely attributable to the strict cut point resulting from the reduced variability achieved through purification using Melon™ gel and SPEAD pretreatment. Consequently, the sensitivity of the screening assay is considered to have been increased.

The screening cut point of this assay was calculated to achieve a 5% false-positive rate and the confirmatory cut point a 1% false-positive rate; however, both positive rates in this clinical trial reached 7.6%. It should be noted that this result is based on only 13 samples with one positive case, indicating insufficient sample size to calculate an accurate frequency. Regarding cut point determination in validation studies, it is common practice to exclude serum samples showing outliers when performing risk-based high-precision evaluations, as recommended in the regulatory guidelines and our validation follows this approach. No such exclusion is applied in clinical trials. Therefore, the presence of patients with interfering molecules like rheumatoid factor, may lead to a higher false-positive rate than the established cut point in clinical trial analyses, which is considered consistent and not contradictory.

5. Conclusion

To reduce interindividual variability and improve drug interference, we developed and validated an ADA analysis method incorporating a two-step pretreatment process. This method uses Melon™ gel to remove nonantibody molecules and SPEAD to remove endogenous drugs from the samples. The validated method demonstrated sufficient sensitivity and drug tolerance for the assay, which was subsequently used in clinical trials of TRK-950.

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Author contributions

Reiji Nishio and Keita Nakanaga conceptualized the study, developed the assay, and collected the data. Ryosuke Yoshinaga conducted the validation study and analyzed the clinical samples. Akira Kurihara oversaw the clinical analyses and curated the clinical trial data. Fumiyoshi Okano supervised the overall project. Reiji Nishio prepared the manuscript, including figures and tables. All authors actively discussed and contributed to the interpretation of the results throughout the study.

Disclosure statement

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Ethical declaration

The authors state that this study was conducted in accordance with the Declaration of Helsinki and International Conference on Harmonization Guidelines for Good Clinical Practice. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Data availability statement

The authors certify that this manuscript reports the secondary analysis of clinical trial data that have been shared with them, and that the use of this shared data is in accordance with the terms agreed upon their receipt. All clinical trial data presented in the article are not publicly available, as they could compromise patient privacy or consent.

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