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Research paper

Development of a competitive binding homogeneous mobility shift assay for the quantification of adalimumab levels in patient serum

Barry D. Hock[a,*], Stewart M. Smith[b], Christopher J. McEntyre[c], Judith L. McKenzie[a], Christiaan Sies[c], Paula E. Keating[b]

[a] Haematology Research Group, Christchurch Hospital, Christchurch, New Zealand
[b] Immunology Section, Canterbury Health Laboratories, Christchurch, New Zealand
[c] Specialist Chemistry Section, Canterbury Health Laboratories, Christchurch, New Zealand

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ABSTRACT

Adalimumab is a TNF specific monoclonal widely used therapeutically. Monitoring adalimumab levels is important for guiding treatment strategies and is predominantly performed using an ELISA. The homogeneous mobility shift assay (HMSA) has many advantages over an ELISA for adalimumab monitoring but current HMSA methodologies do not discriminate between adalimumab and other TNF specific monoclonals such as infliximab. The development and validation of a competitive binding HMSA (cHMSA) specific for adalimumab is reported here. The cHMSA had a lower limit of quantitation of 1.25 μg/ml and the intra-assay and inter-assay coefficents of variation (CV) were < 20%. No signal was detected in adalimumab naïve control serum including those containing rheumatoid factor or infliximab. The majority (14/20) of adalimumab patient samples containing anti-adalimumab antibodies gave a cHMSA signal > 3 standard deviations lower than the controls. The performance of the cHMSA and an ELISA was compared using adalimumab patient samples (n = 82). There was a strong correlation between the assays (r = 0.91) and the intra-class correlation coefficient (0.88) was indicative of good-excellent inter-assay reliability. Bland-Altman plots showed little overall bias and comparison of the subgroups defined using cut-points (1.25 or 7.3 μg/ml) gave percent agreement (> 90%) and Cohens kappa (95% CI: 0.61–0.93) values indicative of substantial-almost perfect agreement. These results demonstrate that cHMSA provides an accurate and specific method for monitoring adalimumab levels and can additionally provide an initial screen for the presence of anti-adalimumab antibodies.

1. Introduction

The use of tumour necrosis factor (TNF) – specific therapeutic monoclonal antibodies such as adalimumab has revolutionized the treatment of chronic inflammatory diseases. However, a substantial proportion of patients either fail to respond to these drugs or subsequently undergo a loss of response (LOR) and relapse (Ben-Horin and Chowers, 2011; Ford et al., 2011; Kalden and Schulze-Koops, 2017). Although adalimumab is a fully humanized antibody, patients can develop anti-adalimumab antibodies (AAA) which may increase drug clearance and/or inhibit their function (van Schouwenburg et al., 2013; Prado et al., 2017). Consequently monitoring of both adalimumab and AAA levels is now considered crucial for treatment optimization (Mitrev et al., 2017; Papamichael et al., 2019b). In patients with a range of inflammatory diseases, therapeutic drug monitoring has been reported to result in improved clinical outcomes and reduced treatment costs in addition to guiding clinical decisions on whether changes should be made to the dose or type of therapeutic (Silva-Ferreira et al., 2016; Ricciuto et al., 2018; Papamichael et al., 2019a,b).

A number of different assay formats exist for the analysis of both adalimumab and AAA with ELISA being the most widely utilised (Ogric et al., 2017). However the solid phase format of ELISA methodologies and the requirement for multiple washing steps may affect both non-specific binding and the affinity of specific interactions. Although mass spectrometry has been utilised for quantification of therapeutic antibodies the lack of an adalimumab specific peptide that's not also found in the serum background precludes its usage for adalimumab monitoring without additional extraction protocols (Ladwig et al., 2017).

Abbreviations: AAA, anti-adalimumab antibodies; AF-adalimumab, Alexa Fluor 488 labelled adalimumab; cHMSA, competitive binding homogeneous mobility shift assay; LOR, loss of response; mAb-AAA, monoclonal anti-adalimumab antibody; SEC, size exclusion chromatography; TNF, tumour necrosis factor

* Corresponding author at: Haematology Research Group, University of Otago, Christchurch, PO Box 4345, Christchurch, New Zealand.
E-mail address: barry.hock@otago.ac.nz (B.D. Hock).

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The homogeneous mobility shift assay (HMSA) provides an alternative assay format that can be used to analyse both drug and anti-drug levels. In this assay system, size exclusion high performance liquid chromatography (SEC-HPLC) is used to quantify the change in molecular weight (MW) that occurs when complexes form between the therapeutic antibody and either antigen or AAA. There are a number of advantages to this system namely that the target-antibody interactions occur in fluid phase and are highly specific, it has a relatively simple single mixture format, there is no requirement for washing steps, and the size of formed complexes can be visualized. HMSA has been utilised for the analysis of both adalimumab and infliximab specific anti-drug levels using fluorescently labelled drug as the probe (Wang et al., 2012, 2013; Hernandez-Breijo et al., 2016; Rubin et al., 2017; Hock et al., 2018). In addition fluorescently labelled antigen (TNF) has been used as a probe to quantify adalimumab and infliximab levels (Wang et al., 2012, 2013; Rubin et al., 2017). The use of recombinant TNF as a probe has the potential limitation that the ratio of trimeric:monomeric TNF present varies according to incubation and storage conditions as well as by binding to adalimumab (van Schie et al., 2016). This, will in turn, affect the size of the complexes formed. In addition, recombinant TNF is relatively expensive to obtain in large amounts and, as a probe, will not discriminate between different TNF binding monoclonals such as adalimumab and infliximab. The poor agreement between the results obtained using HMSA and ELISA methodologies (Steenholdt et al., 2014; Bodini et al., 2015; Clarke et al., 2019) also precludes comparison of cut-points used in different studies.

The recent commercial availability of human monoclonal AAA potentially allows the use of a competitive binding HMSA (cHMSA) for analysis of adalimumab levels. In a cHMSA, samples are incubated with fluorescent adalimumab and AAA which results in formation of high MW complexes of adalimumab bound to AAA. In the presence of competing adalimumab, the proportion of fluorescent adalimumab present as a monomer would then increase in a quantifiable manner. This approach has the advantage that it is specific for adalimumab and so provides accurate quantification even in those patients recently treated with another anti-TNF monoclonal such as infliximab. In addition the assay has the potential to detect the presence of AAA which would, theoretically, decrease the level of monomeric fluorescent adalimumab present.

This report describes the development and validation of a novel cHMSA for the quantitative and specific measurement of adalimumab levels in patient sera. Furthermore the ability of the cHMSA to detect AAA was evaluated.

2. Materials and methods

2.1. Patient and control sera

Patient samples were the residual serum from samples submitted to the diagnostic Immunology Laboratory (Canterbury Health Laboratories, New Zealand) for physician requested tests. The adalimumab samples (n = 82) were a random selection of those submitted for diagnostic testing of adalimumab levels and were blinded during cHMSA analysis. The laboratory is the national referral centre for this testing and therefore the clinical features associated with each sample were unknown. A recent study of samples submitted to the laboratory for analyis of adalimumab or infliximab levels determined that the majority of samples were from patients with inflammatory bowel disease (IBD). The diagnostic analysis of these samples involved determination of adalimumab levels by ELISA (described below) and those with levels < 2 μg/ml were then further analysed for the presence of AAA using a HMSA (Hock et al., 2018). These results were used to subdivide adalimumab samples into AAA^{Pos} (n = 20) or AAA^{Neg} (n = 62). Samples from infliximab treated patients (n = 22) all had serum infliximab levels > 2 μg/ml (range 2–49, mean = 18.5 μg/ml) as determined by diagnostic ELISA (Hock et al., 2016). Diagnostic laboratory samples that had either been tested for the presence of Anti-Saccharomyces cerevisiae antibodies (ASCA) or contained detectable Rheumatoid factor (RF^{Pos}, 35–1880 IU/ml, n = 31) were used as additional controls. Serum from normal donor volunteers (n = 6) was pooled and used for both dilution of standards and the negative control in each assay run as well as for preparation of quality control (QC) samples.

This research was performed as part of the process for introducing a new clinical assay into an International Accreditation New Zealand (IANZ) accredited national laboratory and all patient samples were therefore utilised in accordance with the International Accreditation New Zealand (IANZ) standard NZS/ISO 15189:2012.

2.2. Analysis of adalimumab levels by competitive HMSA

The HMSA methodology was based on a previously described protocol that had been developed in the laboratory (Hock et al., 2018) and utilised fluorescently labelled adalimumab and anti-adalimumab antibody in combination with size exclusion chromatography. Adalimumab was fluorescently labelled in house with Alexa Fluor™ 488 NHS Ester (Molecular Probes, Eugene, OR) as described previously (Hock et al., 2018) and a monoclonal human anti-adalimumab antibody (clone: AbD18655, IgG1) was obtained from Biorad (Hercules, CA).

Serum samples and standards (10 μl) were each added to a well in a 96 well round bottomed plate and diluted by addition of 10 μl PBS. Following addition of 20 μl Alexa Fluor-adalimumab (AF-adalimumab, 3 μg/ml in 0.1% bovine serum albumin (BSA)/PBS) and mixing, 20 μl of anti-adalimumab antibody (3 μg/ml in 0.1% BSA/PBS) was added. Samples were incubated for 1 h at RT to allow immune complex formation prior to addition of 40 μl of 0.1%Tween 20/PBS. Samples (50 μl) were then injected into an HPLC system (Shimadzu Prominence) equipped with a BioSec-3000 column (BioSep™5μmSEC-s3000 290A°, Phenomenex, Torrence, CA) and the chromatography was run at a flow rate of 1 ml/min for 20 min with PBS (pH 7.4) as the mobile phase. The column was operated at ambient temperature (approximately 25 °C). Column eluent was monitored using a fluorescent detector (Agilent 1100 series, 494 nm excitation, 519 nm emission) and raw data collected using Delta 5.5 chromatography data system (Dataworx Pty. Ltd). Peak integration was performed using in house developed, Excel based, analysis that determined the area under each peak as defined using a perpendicular drop from valleys to the baseline.

In order to quantify the amount of Adalimumab present, the area under the 300 kDa peak was used to represent drug-AAA dimer and the area under the 150 kDa peak used to represent free monomeric drug (Fig. 1A–C). The ratio of monomer/ total (monomer + dimer) was determined and normalised against the mean ratio detected in a pool of control serums that were analysed in duplicate during each run. The normalised ratio was defined as arbitrary units (AU). For each run an aliquot of a frozen (-20 °C) adalimumab stock (40 μg/ml in pooled normal serum) was double diluted into pooled normal serum and used to construct a calibration curve. Sample adalimumab concentrations were interpolated from a 4 parameter logistic curve fitted to the AU versus log concentration data (GraphPad Prim version 7; GraphPad software, La Jolla, CA). Any samples with levels higher than the upper standard would be defined as > 40 μg/ml.

2.3. Analysis of adalimumab levels by ELISA

Levels of adalimumab in patient sera were analysed using an in-house developed capture ELISA. The ELISA methodology and performance have been described previously (Hock et al., 2016). In this method drug was captured by plate bound TNF and detected using a biotinylated anti-IgG in combination with avidin-HRP. The lower limit of quantitation (LLOQ) was 0.15 ng/ml and the inter-assay and intra-assay CV's were ≤15%.

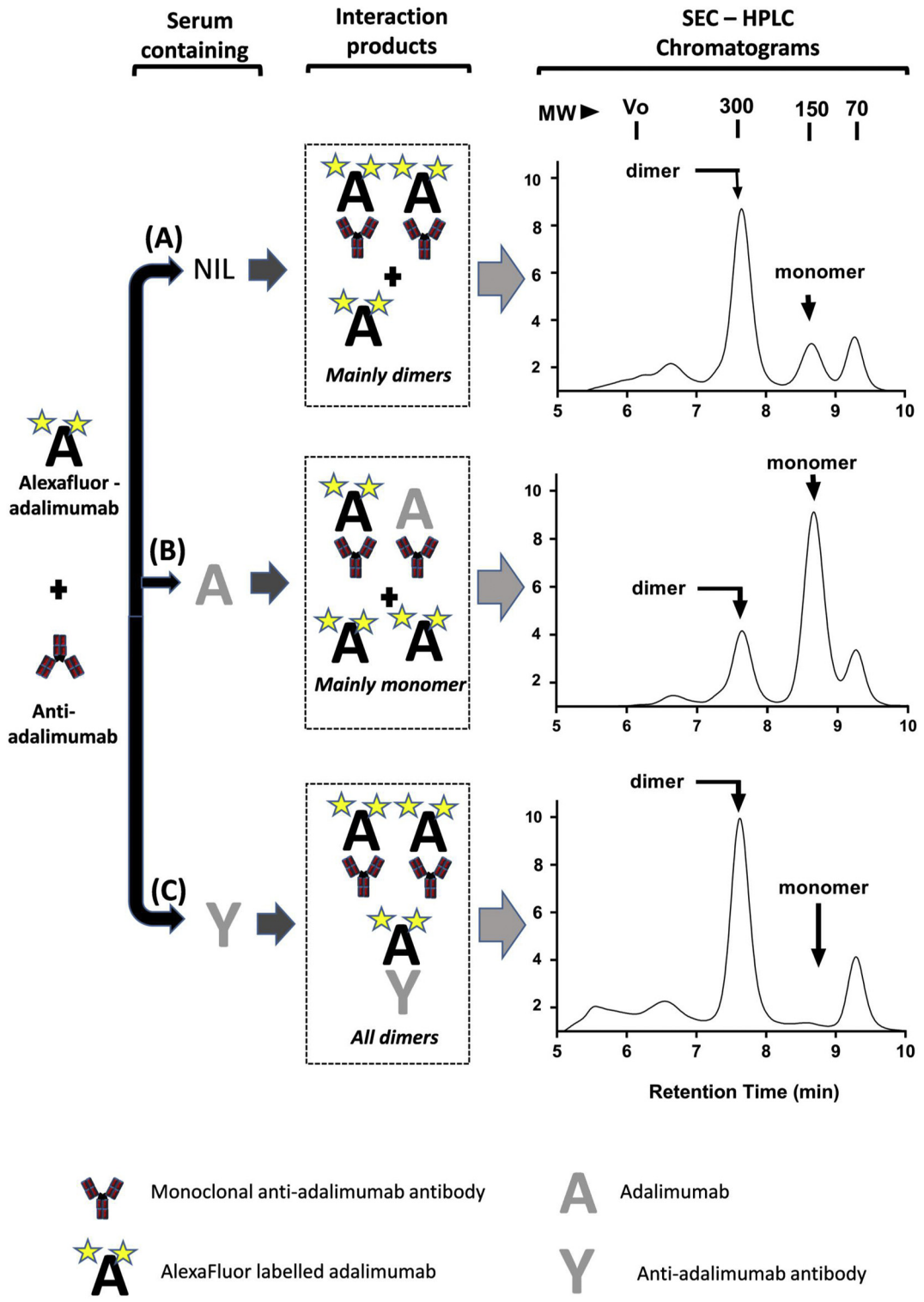

Fig. 1. Schematic illustration of the principle underlying the competitive HMSA assay and representative examples of the chromatograms obtained following SEC-HPLC. Fixed amounts of AF- adalimumab and monoclonal anti-adalimumab antibody are added to serum samples. (A) addition to normal donor serum containing no adalimumab or AAA. The AF-adalimumab is present predominantly in the form of a dimer with a small amount of monomer (B) addition to normal donor serum spiked with adalimumab. The increased competition for binding to the monoclonal anti-adalimumab antibody results in an increased proportion of the AF-adalimumab being present as a monomer as opposed to a dimer (C) addition to patient serum containing anti-adalimumab antibody. The increase in total anti-adalimumab antibody results in all of the AF-adalimumab being present as a dimer. Representative examples of the chromatograms obtained following SEC-HPLC analysis of the respective sample types are shown. MW markers and the position of the monomer and dimer peaks are indicated for each chromatogram.

2.4. Detection of anti-adalimumab antibodies

The presence of AAA in serum samples was determined using HMSA as described previously (Hock et al., 2018). In brief serum samples were acidified/ neutralised then further incubated with AF-adalimumab. Sample were then analysed using an HPLC system in combination with size exclusion chromatography and fluorescent detection. The presence of AAA is reflected in an increase in the amount of AF-adalimumab present as a complex rather than monomer.

2.5. cHMSA validation

The limit of blank (LoB) was determined by measuring drug naïve serum samples ($n = 60$) across multiple runs and then, because many samples had readings ≤ zero, the LOB was calculated non-parametrically as the 95th percentile of the control serum distribution. The Limit of Detection (LoD) was determined as LoB + 1.645xSD where SD was calculated using replicates of patient sera with low levels of signal (1.25–2.5 μg/ml) (Armbruster and Pry, 2008).

The lower limit of quantitation (LLOQ) was determined using back calculated concentrations of the low concentration standards determined over multiple assays. The LLOQ was defined as the lowest concentration that was more than fivefold higher than the LOB and resulted in assay precision (CV, %) and mean bias (% relative error) values < 20% (DeSilva et al., 2003; EMA, 2011).

Assay precision (CV, %) was determined using frozen QC samples prepared by spiking control serum pools with the respective drug at low, mid or high levels. The QCs were then analysed across multiple plates. Parallelism was analysed by performing serial dilutions of patient sera with high adalimumab levels and then determining whether the dilution corrected concentrations had a %CV < 20% (DeSilva et al., 2003; EMA, 2011). Dilutional linearity was demonstrated by performing serial dilutions of control sera spiked with high levels of adalimumab and then determining whether the dilution corrected concentrations had a %CV < 20% (DeSilva et al., 2003; EMA, 2011).

2.6. Statistical methods

Sera from patients receiving adalimumab were analysed by both cHMSA and ELISA and the results compared. Qualitative inter-assay reliability was assessed using % agreement and Cohens Kappa. The kappa value was categorised (Landis and Koch, 1977), based on the 95% CI, as 'substantial' (0.61–0.8) and 'almost perfect' (> 0.81).

The correlation, reliability and agreement between the quantitative results of the assays was assessed using data from those samples which had adalimumab concentrations > LLOQ in the cHMSA.

Correlation was assessed using scatter plots and pearsons correlation coefficient determined.

Quantitative agreement between the assays was assessed using a Bland-Altman plot in which, for each pair of sample measurements, the percentage difference between the measurements is plotted against the average of the two measurements. The mean % difference and its 95% CI are then calculated together with the limits of agreement (mean ± 1.96 x sd).

The inter-assay reliability was assessed using the intraclass correlation coefficient (ICC) which was calculated using a single measurement, absolute agreement, two-way mixed effects model. The reliability of the ICC was categorised (Koo and Li, 2016) based on the 95% CI as 'substantial' (0.61–0.8) and 'almost perfect' (> 0.81).

Cohens Kappa and ICC estimates were calculated using a SPSS statistical package (IBM SPSS Statistics, Version 25). All other analysis and graphing was performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla, California USA.

3. Results

3.1. Assay principles

The competitive binding assay was based on the underlying assumptions that (i) in a solution containing AF-Adalimumab and monoclonal anti-adalimumab antibody (mAb-AAA) the AF-adalimumab would, at equilibrium, be present as a monomer and/or a higher MW complex with AAA (ii) in samples containing adalimumab or AAA the equilibrium would be shifted so that the proportion of monomeric AF-adalimumab increased or decreased respectively (iii) the proportion of AF-adalimumab present as a monomer or in a complex can be quantitatively determined by SEC-HPLC.

In order to quantify adalimumab using cHMSA the amount of AF-adalimumab must be in excess of the mAb-AAA (Zettner, 1973). Preliminary experiments (data not shown) determined that the addition of these reagents at a 1:1 ratio resulted in AF-adalimumab being present predominantly as a heterodimer with a small amount of excess AF-adalimumab monomer detectable.

The principles of the assay are outlined in Fig. 1A-C. In brief a fixed amount of AF-Adalimumab is first added to the sample and then, following mixing, the mAb-AAA is added at a 1:1 ratio. During subsequent incubation an equilibrium is established and the MW of the AF-adalimumab is then determined by SEC-HPLC.

In control sera (Fig. 1A) the AF-adalimumab would be expected to be present predominantly as a dimer with a smaller amount of monomer. The corresponding chromatogram showed a large peak corresponding to the expected MW (300 kDa) of an IgG dimer (AF-Adalimumab + mAb-AAA) and a smaller peak corresponding to monomeric AF-Adalimumab (150 kDa).

Adalimumab spiked into the same serum (Fig. 1B) would compete with the AF-adalimumab for binding to the mAb-AAA and therefore be expected to increase the proportion of monomeric AF-adalimumab. Consistent with this, the corresponding chromatogram shows a reduction in the size of the 300 kDa dimer peak and a concomitant increase in the size of the 150 kDa monomer peak.

Serum that lacks detectable adalimumab but contains AAA (Fig. 1C) would be expected to shift the equilibrium toward the increased formation of dimer. The corresponding chromatogram showed disappearance of the monomeric AF-Adalimumab (150 kDa) and a corresponding increase in the size of the 300 kDa dimer peak.

3.2. Quantification of adalimumab by competitive HMSA

The proportion of AF-adalimumab present as a monomer rather than a dimer in serum samples directly reflects the amount of adalimumab present that can compete for binding to mAb-AAA.

In order to quantify the amount of Adalimumab present, the areas under the dimer and monomer peaks of AF-adalimumab associated fluorescence (Fig. 1A–C) was determined and the ratio of monomer/(monomer + dimer) areas then calculated. Standards were diluted in a normal serum pool and the mean ratio observed ranged between 0.91 (range 0.88–0.93) in the 40 μg/ml standard and 0.18 (range 0.07–0.40) in the 0 μg/ml standard. For each experiment, the ratios of both standards and samples were normalised against the mean ratio of the respective 0 μg/ml standard and the resulting normalised ratio defined as arbitrary units (AU).

The ratio of monomer in sera from all adalimumab naïve controls tested ($n = 67$) was similar to that of the normal serum pool and the normalised ratio (Fig. 2A) was therefore tightly clustered around one (mean ± sd = 0.99 ± 0.22, range 0.57–1.63 AU). No outliers suggestive of false positives were observed in any of the control groups including RF^{Pos} and infliximab containing sera.

Serum from patients receiving adalimumab had been previously analysed in a diagnostic laboratory. Those samples with low adalimumab levels, as determined by ELISA, had been further analysed for

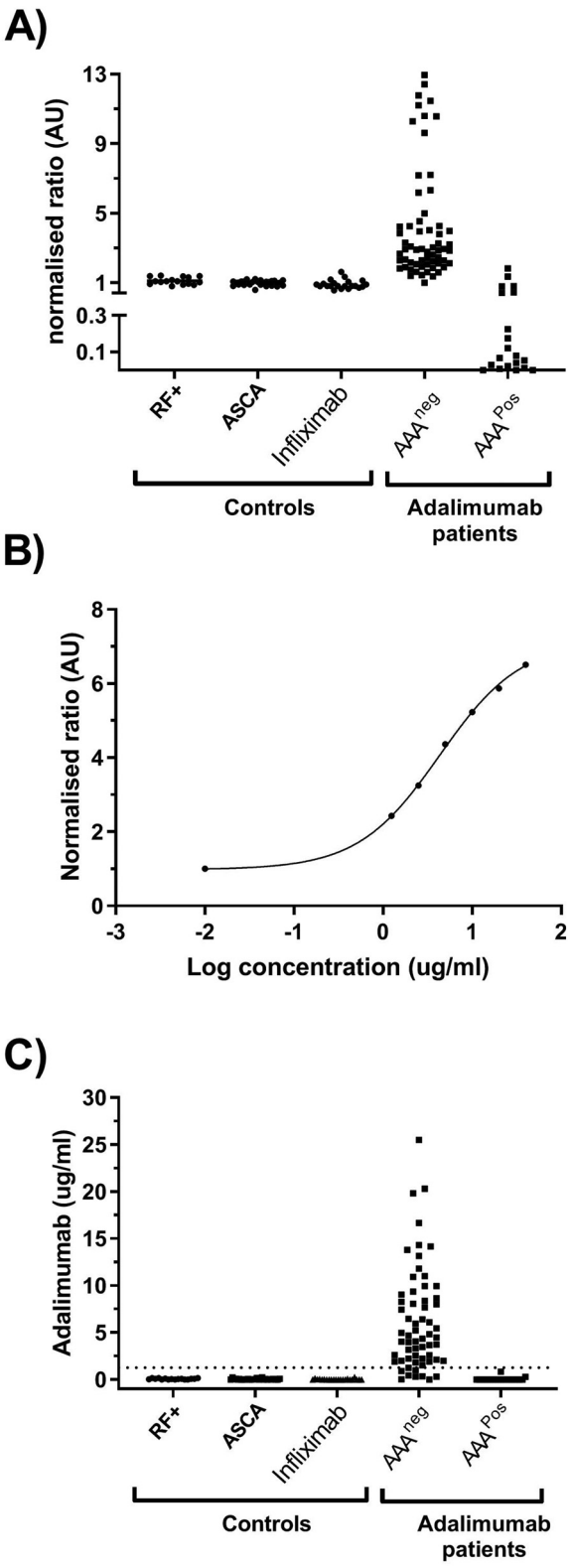

Fig. 2. Serum levels of adalimumab as determined by cHMSA. Sera from both patients receiving adalimumab and controls were analysed by cHMSA. Data obtained following analysis of chromatograms was then used to (A) Determine the normalised ratio of monomer / (monomer plus dimer) peaks. The normalised ratio was expressed as AU and shown as a scatter plot for each patient and control grouping. (B) Generate a standard curve by plotting the normalised ratio versus log concentration (C) Determine the adalimumab concentration corresponding to the AU of each serum sample. Concentrations were interpolated from the respective standard curve and data shown as a scatter plot of μg/ml adalimumab for each patient and control grouping. The dotted line indicates LLOQ. Control samples included sera submitted to the diagnostic laboratory for analysis of ASCA (n = 29), sera known to contain RF (n = 16) and sera containing infliximab (n = 22). Adalimumab patients were subdivided into negative (n = 62) and positive (n = 20) subsets based on whether their sera contained detectable anti-adalimumab antibodies (AAA).

the presence of anti-drug antibodies. Based on those results samples were divided into those which were positive in the AAA assay (AAA^{Pos}) and those which were either negative in the AAA assay, or untested, because of their higher drug levels (AAA^{Neg}). The normalised ratios in AAA^{Neg} samples ranged between 1 and 13 AU (mean = 4. 2). In contrast 14/20 (70%) AAA^{Pos} samples had normalised ratios < 0.3 AU, which is itself > 3 standard deviations lower than the mean of the control data.

Calibration curves based on the AU values were constructed using standards in the range 1.25–40 μg/ml (Fig. 2B). The back calculated concentrations of the standards used for 9 calibration curves was determined and for each standard the mean bias was < 15% of the nominal values and the %CV < 13%. These calibration curves were then used to determine the adalimumab concentration in control and patient sera (Fig. 2C). Levels in drug naïve patient samples were low (range 0–0.26, mean = 0.06 μg/ml) and based on this data the LOB was calculated as 0.16 μg/ml and the LOD as 0.36 μg/ml. The lowest standard (1.25 μg/ml) was defined as the LLOQ as its intra-assay CV (10%), inter-assay CV (5%) and mean bias (10%) were all < 15%.

Precision was assessed using QCs analysed over 9 separate runs. The inter-assay CVs of the QC were in the range 2–12% (mean = 7.9%) and the intra-assay CVs were in the range 2.8–12% (mean = 7%).

Parallelism between the standard curve and serial dilutions of patient samples was assessed using 3 patient samples which had high levels of adalimumab as determined by ELISA. The CVs for the dilution corrected concentrations determined for each sample were < 20% and therefore demonstrated acceptable parallelism (Fig. 3A). Dilution linearity was also assessed using serial dilutions of serum spiked with adalimumab at a concentration (70 μg/ml) higher than the top standard (40 μg/ml). The dilution corrected concentrations were all within 20% of the nominal concentration and the CV was < 20% demonstrating the absence of a prozone effect (Fig. 3B).

Serum from patients receiving adalimumab had drug levels ranging from undetectable to 25 μg/ml. The levels in the AAA^{Pos} subset were all below the LLOQ (range = 0–0.8 μg/ml, mean ± sd = 0.05 ± 0.2 μg/ml). Levels in the AAA^{Neg} subset ranged from 0 to 25 μg/ml (mean ± sd = 6.2 ± 5.4 μg/ml) with 53/62 (85%) samples having levels > LLOQ.

3.3. Comparison of ELISA and cHMSA

The results obtained following cHMSA analysis of sera from adalimumab treated patients was compared with those obtained for the same samples by a diagnostic laboratory using an ELISA. Of the 82 sera analysed, 29 had levels below the LLOQ (1.25 μg/ml) of the cHMSA. Of these 29 samples, 26 were also < 1.25 μg/ml in the ELISA, Therefore with respect to identifying samples with low levels of adalimumab (< 1.25 μg/ml), the % agreement was 94% and the Cohens kappa (95% CI: 0.76–0.98) categorised the agreement as substantial-almost perfect.

Further comparison was limited to the 53 samples with levels above LLOQ in the cHMSA. There was a strong correlation (r = 0.91) between the assays (Fig. 4A).

The level of agreement between the two assays was assessed using a Bland-Altman plot (Fig. 4B). The scatter plot showed the percentage differences between the assays had an approximately normal distribution and did not vary across the concentration range. The mean

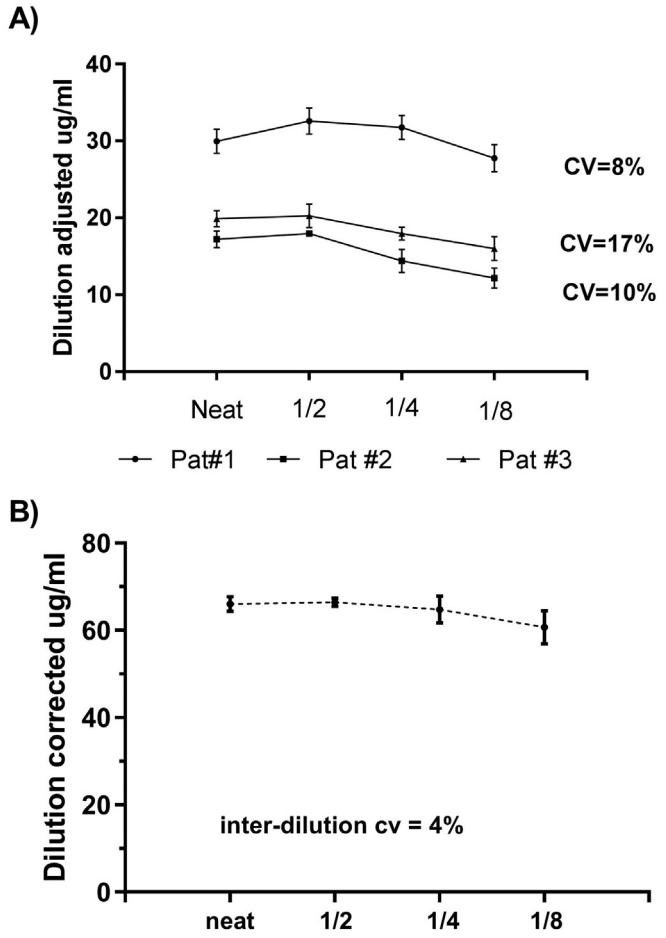

Fig. 3. Dilutional linearity and parallelism of adalimumab containing samples (A) Parallelism was assessed using normal serum spiked with adalimumab and then serially diluted in normal serum. Duplicates of each dilution was analysed by HMSA and results shown as a plot of the dilution corrected concentrations. The inter-dilution CV using the mean of each duplicate is shown. Data are from a representative experiment (B) Dilutional linearity was assessed using patient sera (n = 3) containing high levels of adalimumab that was serially diluted in normal serum. Duplicates of each dilution was analysed by HMSA and results shown as a plot of the dilution corrected concentrations (mean ± SEM). For each patient sample the inter-dilution CV calculated using the mean of each duplicate is shown. Data are from a representative experiment.

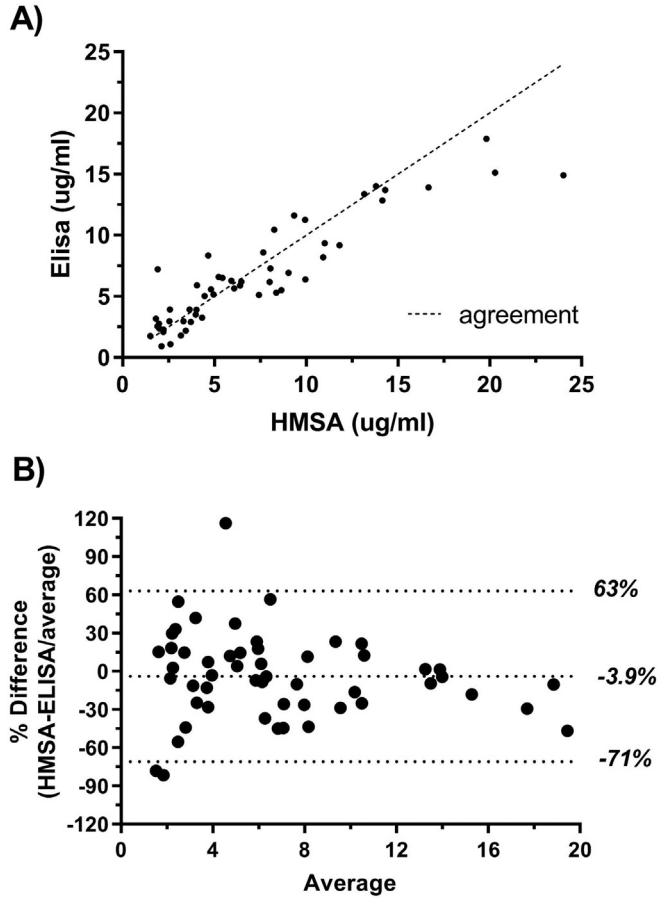

Fig. 4. Comparison of adalimumab concentrations determined by cHMSA and ELISA. Results obtained using cHMSA and ELISA were compared in patient sera which had adalimumab levels > LLOQ in the cHMSA assay (A) Scatter plot of μg/ml adalimumab detected by ELISA versus cHMSA. The line of agreement is shown as a solid line (B) Bland-Altman plot comparing the ELISA and cHMSA. For each sample the difference between the results is expressed as a percentage of their average and then plotted against their average. The dotted lines and associated numbers indicate the mean and 95% limit of agreement for the percentage difference.

percentage difference was −3.9% (95%CI -13.3-5.5) indicating little overall bias. The limits of agreement indicated that for 95% of the samples the percentage difference was between 71% lower and 63% higher than the average.

The overall inter-assay reliability was analysed using the ICC. The ICC value (0.88, 95% CI = 0.80–0.93) was indicative of good-excellent reliability.

A previous clinical study using the same ELISA utilised in this study suggested a threshold of 7.3 μg/ml for discriminating those inflammatory bowel disease patients with active disease (Barclay et al., 2019). Using the same cut-point in the current study, 17/82 samples had levels > 7.3 μg/ml by ELISA. The cHMSA identified 22 samples as having levels > 7.3 μg/ml including 16 of the 17 identified by ELISA. The level of agreement between the assays with respect to this cut-point (% agreement = 91%, Cohens kappa 95% CI: 0.61–0.93) was categorised as substantial-almost perfect (Landis and Koch, 1977).

4. Discussion

Therapeutic drug monitoring is now widely used to guide treatment decisions in patients receiving anti-TNF agents such as adalimumab (Ben-Horin and Chowers, 2014; Silva-Ferreira et al., 2016; Mitrev et al., 2017). Currently a number of different assay types are utilised for the quantification of adalimumab with ELISA based methodologies being the most common (Ogric et al., 2017). The recent commercial availability of human monoclonal AAA provides the opportunity to develop a cHMSA for the analysis of adalimumab. This type of assay has a number of potential advantages over an ELISA including the occurrence of the target-antibody interactions in fluid phase and a relatively simple, single mix, setup. Additionally cHMSA unlike current HMSA methodologies, detects only adalimumab in patients undergoing a change of therapy due to LOR. This study analysed the ability of cHMSA to quantify serum adalimumab levels and compared the results with those obtained using a conventional ELISA.

The validation of the cHMSA showed it had the required performance and reliability for use as an analytical method. The cHMSA had a LLOQ (1.25 μg/ml) similar to that of the most widely used HMSA methodology (Rubin et al., 2017). Although ELISA methodologies have a much lower LLOQ (Ogric et al., 2017) there is no clinical necessity for the increased sensitivity given that therapeutic cut-points are in the range 5–12 μg/ml (Mitrev et al., 2017). Direct comparison of ELISA and cHMSA results in this study demonstrated high levels of both qualitative and quantitative agreement. Given that these assays were

performed in separate laboratories this provides strong evidence that cHMSA and ELISA have similar performances and can be considered interchangeable. A limitation in this comparison is that the ELISA utilised was developed for use in the analysis of either adalimumab or infliximab levels and therefore detects both drugs, unlike the cHMSA which is adalimumab specific. Consequently samples from patients who have recently changed from infliximab to adalimumab may contain both drugs and therefore the ELISA may report a substantially higher adalimumab concentration than the cHMSA. We were unable to access relevant clinical records and therefore could not accurately determine how many of such samples were present. However the data presented in Fig. 4a together with the absence of overall bias suggests few, if any, such samples were present. The identification and removal of such samples would be expected to increase the level of agreement between these assays and, given that the level of agreement was already high, would not affect the current study's findings.

In contrast to this study's findings, poor agreement between HMSA and ELISA methodologies has been reported with respect to quantification of both adalimumab (Bodini et al., 2015; Clarke et al., 2019) and infliximab (Steenholdt et al., 2014). The HMSA methodology does however differ markedly from the cHMSA in that it uses direct binding of fluorescent TNF to detect adalimumab. The performance of the HMSA may therefore be impacted by storage, incubation and adalimumab induced changes in the ratio of trimeric:monomeric TNF present (van Schie et al., 2016).

Patients receive adalimumab as a treatment for autoimmune diseases, particularly IBD and arthritis. The specificity of the cHMSA was therefore analysed using sera from patients having, or being investigated for, the presence of autoimmune disorders. The presence of rheumatoid factors is a marker of autoimmune disease and known to be a major cause of false positives in many immunoassays (Ward et al., 2017). RF^{Pos} samples together with samples tested for the IBD associated marker ASCA gave only background signal in the cHMSA. Patients receiving adalimumab are often switched to/from the therapeutic infliximab which is also a TNF specific IgG1 mAb. Samples with detectable infliximab did not generate any positive signals in the cHMSA further confirming the assays specificity.

Analysis of AAA levels normally requires a separate assay. However the ability of cHMSA to determine the size of the fluorescence associated molecule(s) potentially allows the assay to detect both adalimumab and AAA. This arises because in order for the cHMSA to have the required sensitivity and dose response, the amount of labelled ligand (AF-adalimumab) must be in excess of the target (mAb-AAA) (Zettner, 1973). Therefore in control samples an equilibrium is established where a proportion of the labelled ligand is not bound to target and can be clearly identified as monomeric based on its MW. The presence of competing ligand (i.e adalimumab) would then shift the equilibrium toward increased proportions of unbound labelled ligand. However the presence of increased target (i.e. AAA) would have the opposite effect and shift the equilibrium so that the proportion of unbound labelled ligand decreases. In the current study analysis of patient samples known to contain AAA confirmed that, in the majority of samples the presence of AAA markedly decreased the proportion of unbound labelled ligand. This decrease was not however observed in all samples that were AAAPos using an established AAA specific assay. This may reflect the fact that the AAA specific assay is drug tolerant and therefore can detect bound, as well as excess, unbound AAA. Additionally, low affinity and/or low concentration AAA may be more detectable in the specific assay as the cHMSA relies on the serum AAA being able to shift the equilibrium established using a high affinity (K_D = 0.06 nM) commercial AAA. It is currently unclear whether the additional AAA that can be detected using drug tolerant assays have any clinical relevance (Mitrev et al., 2017) and therefore larger studies are required to determine whether the cHMSA format has sufficient sensitivity to be used for the detection of clinically relevant AAA. Irrespective of those findings, it is clear that in addition to determining adalimumab concentrations, the cHMSA also provides a useful initial screening tool for the presence of AAA.

An alternative approach for specific quantification of adalimumab using HMSA would be to fluorescently label the commercially available monoclonal AAA and then use this reagent to directly bind adalimumab. This has the advantage that it requires only a single reagent and is likely to have similar assay performance. The major advantage of the cHMSA is that it can also be used to screen for the presence of AAA which are undetectable in a direct binding format. In addition the cHMSA utilizes the same fluorescent reagent (i.e. AF-adalimumab) utilised for HMSA based AAA quantification. Therefore large amounts of AF-adalimumab can be prepared from clinical stocks relatively cheaply and then used for both drug and anti-drug assays.

The results of this study demonstrate that the cHMSA has the required performance, reliability and specificity to be used as an analytical method for the detection of adalimumab in patient sera. This novel approach has a number of advantages over existing methodologies including its relatively simple single mix setup, the occurrence of the antibody interactions in fluid phase, its specificity for adalimumab and its ability to identify the majority of AAA^{Pos} samples. The major limitation of this approach is the requirement for access to HPLC facilities.

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