RESEARCH ARTICLE

Analytical validation of an in-house method for adenosine deaminase determination

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Background: The adenosine deaminase (ADA) enzyme is a marker of inflammatory processes whose activity can be measured through a colorimetric method developed as an in-house assay. This validation can reduce costs and expand the alternatives for laboratory diagnosis.

Methods: The ADA analysis was achieved through a modified form of Giusti and Galanti's (1984) method. The following parameters were characterized: calibration curve, linearity, analytical sensitivity, limit of detection, limit of quantification, method working range, precision (within-assay and between-assay), bias, total analytical error, and sample stability. The results were statistically evaluated and compared with quality specifications based on biological variations and the performance of commercial tests.

Results: The analytical sensitivity and limit of detection (0.013 and 3.0 U/L, respectively) were lower than those of commercial tests. The method's working range was 3.2–100.0 U/L. According to the quality specification, the method showed optimum performance with a bias <3.5%. However, repeatability (2.2% and 1.7% for normal- and high-activity samples, respectively) and reproducibility achieved worse results when compared to commercial tests. The method demonstrated an inappropriate between-assay precision for low enzymatic activity (10.4%) and the minimum and desirable performance for medium (8.8%) and high (5.0%) activities, respectively. It also presented at least a minimum performance (<25%) for the total analytical error of the three analyzed samples. The pleural fluid samples were found to be stable at –20°C for six days.

Conclusion: The findings show that the in-house method displays an acceptable performance and is capable of generating results comparable to existing commercial tests.

KEYWORDS

adenosine deaminase, enzyme measurement, standardization, total quality management, validation studies

1 | INTRODUCTION

The measurement of adenosine deaminase (ADA) activity in pleural fluid is recommended as a useful routine diagnostic test when investigating tuberculous pleuritis. The culture of *Mycobacterium tuberculosis*, the gold standard for diagnosis, has a long turnaround time. In addition, the direct detection of acid-fast bacillus has a low sensitivity when compared to the ADA activity measurement.^{1,2}

ADA is a signaling molecule related to the activation of T lymphocytes, making it a useful inflammation marker,³ especially for meningitis and tuberculous pleuritis.⁴ A laboratory determination of ADA activity is simple, quick, and relatively cheap. It can be performed on several types of biological samples—such as pleural, peritoneal, pericardial, and cerebrospinal fluids, as well as serum—depending on the clinical suspicion. Indeed, the ADA test is currently recommended as an additional diagnostic method for tuberculous pleuritis.^{5,6}

Among the methods for ADA activity evaluation, Giusti and Galanti's (1984) colorimetric method is the most commonly applied. This is based on the detection of ammonia formed by ADA action on adenosine. Ammonia is quantified by a reaction with phenol, which generates an indophenol derivative with an intense blue

color (Figure 1B) that can be spectrophotometrically quantified.^{7,8} This method is often performed through manual or automated techniques, and it can be fully produced by the laboratory itself (ie, it is an in-house method).

There are two available tests for an ADA enzymatic/colorimetric assay registered with the Brazilian Health Regulatory Agency (Anvisa): Biosystems SA of Spain (Figure 1C) and Ebram Produtos Laboratoriais Ltda, SP of Brazil (Figure 1D). The latter is based on the enzymatic deamination of adenosine into inosine, which is then converted to hypoxanthine before becoming uric acid and hydrogen peroxide, the latter being monitored kinetically (Figure 1D).^{9,10} These commercial tests are expensive, however, hindering their routine use in smaller laboratories and countries with limited resources.

A validation of a proposed in-house method aims to describe its analytical performance through statistical analysis in order to obtain reliable estimates for how well it meets clinical needs. This should naturally be performed before introducing it into the diagnostic routine.^{11,12}

A validation study requires the use of acceptability criteria. Validation tests need to have goals for their results, because these are useful when evaluating the performance of a method.

FIGURE 1 Different methods for measurement of ADA activity. (A) Enzymatic deamination of adenosine. (B) Basis of the in-house method (modified from⁸). (C) Basis of the Biosystems® method, Spain.⁹ (D) Basis of the Ebram® method, Brazil¹⁰

Quality specifications based on biological variations take into consideration the physiological fluctuation of the analyte around its homeostatic point. It can be obtained from the intra- and inter-individual components, and these are expressed as coefficients of variation (CV) (specifically CV_i and CV_g, respectively). From this, the allowable imprecision, bias and total analytical error (TAE) can be calculated.¹³

To validate an in-house method, the literature recommends evaluating the accuracy, precision, measuring interval, sensitivity, specificity, linearity, sample stability, and reference interval (reference values), as well as studying the interferences.^{11,12,14} It is also recommended for a laboratory to keep records of specifications of a method's performance while the method is in use and for at least two years after its discontinuation.¹²

Finally, the validation of an in-house method can provide benefits in the form of cost reduction and less waste and rework, because practitioners can confidently decide to introduce it into their laboratory routine.

In order to reduce costs by offering an in-house manual method for determining ADA activity, we have performed studies to validate the laboratory test in pleural fluid (PF) samples. We compare the results of this with those obtained using a commercial kit, which can be considered a reference point for our biochemical study.

2 | MATERIALS AND METHODS

2.1 | Materials

Adenosine AG (analytical grade), phenol USP (United States Pharmacopeia), monobasic sodium phosphate AG, dibasic sodium phosphate AG, and ammonium sulfate AG were chemicals sourced from Labsynth® (Diadema, Brazil). Sodium hypochlorite 5% and sodium nitroprusside AG came from Dinamica Química Contemporânea Ltda® (Diadema, Brazil). The commercial test (QUIMIADA—Adenosine Deaminase) and the ADA calibrator 50.8 U/L came from Ebram® (São Paulo, Brazil).

Residual pleural fluid specimens were used as samples. This study was registered with the Brazilian National Ethics Committee of Human Research (80788317.0.0000.0121), and it conforms to the US Federal Policy for the Protection of Human Subjects.

The analyses were performed using a UV-1203 Shimadzu Spectrophotometer (Kyoto, Japan) and an Ethik technology water bath (Vargem Grande Paulista, Brazil).

2.2 | Methods

This validation study was developed at the clinical analysis laboratory of the University Hospital of the Federal University of Santa Catarina.

The in-house method was conducted as follows: 500 µL of 50 µmol/L sodium phosphate buffer pH 6.5 (B), 500 µL of buffered adenosine (21 mmol/L adenosine in 50 mmol/L phosphate buffer

pH 6.5) as sample blank (SB), 500 µL of buffered adenosine (for sample S), and 300 µL of phosphate buffer mixed with 200 µL of 0.15 mmol/L ammonium sulfate solution (standard P) were incubated at 37°C for 2 minutes in a water bath.

Subsequently, 20 µL of the sample was added to the S-tube and incubated at 37°C for 60 minutes in a water bath. Following this, the reaction was interrupted by the addition of 1 mL of phenol/sodium nitroprusside solution (106/0.17 mmol/L) to all tubes. Furthermore, 20 µL of the sample was added to the SB-tube, and 1 mL of 11/125 mmol/L alkaline solution (NaOCl/NaOH) was added to all tubes. After mixing, the tubes were incubated at 37°C for 30 minutes in a water bath. The absorbance was set at 630 nm.

The subsequent study carried out to validate this in-house method is briefly described below:

2.2.1 | Calibration curve

The calibration curve was performed in duplicate using the ammonium sulfate standard (0.150 mmol/L) in dilutions that correspond to enzymatic activities of 10.0, 20.0, 40.0, 60.0, 80.0, and 100.0 U/L, which go throughout the linearity range. In order to check its correspondence with the calibrator, a calibration curve was prepared using the ADA® calibrator with the following final enzymatic activities analyzed in duplicate: 5.8, 10.2, 20.3, 30.5, 40.6, and 50.8 U/L.

2.2.2 | Linearity

The dosage of ammonium was performed using an ammonium sulfate solution at concentrations of 0, 3, 6, 12, 18, 30, 60, 120, 180, 240, and 300 µmol/L. These correspond to ADA activities of 0, 10, 20, 40, 60, 100, 200, 400, 600, 800, and 1000 U/L, respectively. Tests were performed three times in duplicate. Linearity was assessed through a visual inspection of the plot, which was examined for outliers, and the residuals plot (ie, the difference between the observed y value and the y value predicted by the equation line for each x value). The distribution of residuals around zero confirms linearity.¹¹

2.2.3 | Analytical sensitivity

According to the Eurachem Guide,¹¹ the analytical sensitivity is the change in instrument response that corresponds to a proportional measured quantity. This can be obtained through the equation line produced from the calibration curve ($f(x) = Ax + B$), where the angular coefficient (A) corresponds to the analytical sensitivity.

2.2.4 | Limit of detection and limit of quantification

The limit of detection (LOD) was determined through the formula $LOD = 3 \cdot SD'_0$, where SD'_0 represents the standard deviation of 10 replicates of PF blank samples divided by the root square of the number of replicates.¹¹

Calibration curve

FIGURE 2 Calibration curves of the in-house method for ADA activity determination using ammonium sulfate standard solution and Ebram® calibrator standard

The limit of quantification (LOQ), meanwhile, was determined through the formula $LOQ = K \cdot SD'_0$, where constant K is defined as 5 according to the Eurachem Guide.¹¹

2.2.5 | Method working range

The method working range (MWR) was established as the interval between LOQ and linearity, according to the Eurachem Guide.¹¹

2.2.6 | Within-assay precision (repeatability)

As recommended by the Eurachem Guide,¹¹ the study was performed using the same equipment and with the same reagent, lot, and operator over a short period. Two PF samples with low (26 U/L) and high (120.5 U/L) activities were analyzed with ten replicates in two moments of the same day, generating 20 results for each level. The repeatability standard deviation (SD_R) was obtained by taking the square root of the within-assay mean square (the sum of difference's squares divided by the degrees of freedom [the number of measurements minus one]). The coefficient of variation, CV_R , was calculated as the ratio of the standard deviation to the average and expressed as a percentage.

2.2.7 | Between-assay precision (reproducibility)

For the reproducibility study, there is intermediate uncertainty because it was performed in a single laboratory. PF sample aliquots with three different activities (low = 5.9 U/L, medium = 23.5 U/L, high = 76.3 U/L) were analyzed five times for 10 days.¹¹ The within-laboratory reproducibility standard deviation (SD_{WL}) was calculated by taking the sum of difference's squares and dividing it by the degrees of freedom (the number of measurements minus one). The coefficient of variation, CV_{WL} , was calculated as the ratio of the standard deviation to the average and expressed as a percentage.

2.2.8 | Bias

A PF sample with low (6.1 U/L) enzymatic activity was selected for a recovery study in accordance with the Eurachem Guide.¹¹ The sample was divided into three aliquots, and the calibrator (ADA® 50.8 U/L) was added to each one of them in order to obtain activities of 17.3, 28.5, and 39.6 U/L (low, medium, and high, respectively). Each sample was analyzed five times over five days, and the mean activity for each was compared with the expected value. The formula of %Recovery = (obtained mean value/expected value)*100 was used to calculate the apparent recovery.

2.2.9 | Total analytical error

The TAE was estimated using the formula $TAE = |\text{bias}| + Z \cdot CV$,¹³ where bias (expressed as a percentage) corresponds to the systematic error percentage, estimated here via the recovery study; Z is the multiplier value related to the level of confidence (1.96 for 95% of confidence); and CV is the percentage of random error (standard deviation), estimated here through the between-assay precision study (CV_{WL}).¹³

2.2.10 | Sample stability

Three pools of PF samples with low (4.5 U/L), medium (26.1 U/L), and high (68.1 U/L) activities were prepared and divided into three aliquots. One aliquot of each level was stored at room temperature (22–26°C), one was refrigerated (2–8°C), and one was frozen (–20°C). Each aliquot was subdivided into eleven "test aliquots," which were later analyzed in duplicate 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, and 14 days afterward. The daily average results were compared to the one at time zero.¹⁴ For this assay, an 8.8% variation was considered acceptable according to the quality specifications for imprecision.¹⁵

TABLE 1 Characteristics of linearity, sensitivity, LOD, and LOQ of the in-house method compared to the commercial tests available in Brazil

Characteristics Method	Linearity (U/L)	Sensitivity (U/L)	Limit of detection (U/L)	Limit of quantification (U/L)
In-house	100	0.013	0.2	3.2
Ebram [®]	200	0.033	NA	NA
BioSystems [®]	150	NA	1.65	NA

NA, Not available.

TABLE 2 Performance characteristics of the in-house method compared to the commercial tests available in Brazil and specifications for analytical quality for ADA

Method Performance	ADA activity in the sample	In-house	Ebram ^{®a}	BioSystems ^{®b}	Analytical quality based on biological variation ^c
CV _R (%)	Low	NA	1.47	NA	NA
	Medium	2.2	1.45	1.6	
	High	1.7	NA	1.2	
CV _{WL} (%)	Low	10.4	4.9	NA	Minimum ≤8.8
	Medium	8.8	2.0*	4.1	Desirable ≤5.9
	High	5.0	NA	4.4	Optimum ≤2.9
BIAS(%)	Low	-1.3	NA	NA	Minimum ≤10.5
	Medium	-1.9	2.3	NA	Desirable ≤7.0
	High	-0.6	2.3	NA	Optimum ≤3.5
TAE(%)	Low	18.4	NA	NA	Minimum ≤25.0
	Medium	16.4	NA	NA	Desirable ≤16.7
	High	8.8	NA	NA	Optimum ≤8.3

CV_R, repeatability coefficient of variation; CV_{WL}, within-laboratory coefficient of variation; TAE, total analytical error; NA, Not available.^aData from Ebram[®] test¹⁰: CV_R for 11.11 and 30.74 U/L, CV_{WL} for 9.63 and 29.62 U/L, and bias for 52.3 and 201.9 U/L.^bData from BioSystems[®] test⁹: CV_R and CV_{WL} for 26.7 and 63.8 U/L.^cPerformance specifications for analytical quality based on biological variation, calculated according to Ricós¹⁵ using 11.7% and 25.5% as CV_i and CV_g, respectively.

2.2.11 | Comparison of methods

An assessment of the in-house method was achieved by comparing it with the equally manual QUIMIADA (Ebram[®]) method, in accordance with the literature.¹⁶ In brief, 40 PF samples comprising the MWR were analyzed using both methods. The results were then evaluated using the Bland-Altman model and Passing-Bablok regression analysis.

2.3 | Statistical analysis

Data capture and analysis were performed using Microsoft[®] Excel with the help of the Analyse-it software for Microsoft[®] Excel (version 4.95).

3 | RESULTS

3.1 | Calibration curve

The calibration curve obtained with the ammonium sulfate standard solution ($y = 0.0133x - 0.0103$) displayed a coefficient

of determination (R^2) of 0.9979. This was similar to that obtained for the calibrator ($y = 0.0133x - 0.002$), with (R^2) of 0.9997 (Figure 2).

3.2 | In-house method performance

The performance results for the in-house method are shown in Tables 1 and 2. Figure 3 presents the results of the linearity study with dilutions corresponding to activities ranging from 0 to 1000 U/L.

The in-house method for the enzymatic activity of ADA showed an MWR ranging from 3.2 to 100 U/L (Table 1).

The repeatability of the in-house method, as evaluated through the CV_R, was 2.2% and 1.7% for normal- (26.0 U/L) and high-activity (120.5 U/L) samples, respectively (Table 2). The reproducibility, given by CV_{WL}, was 5.0%, 8.8%, and 10.4% for samples with high (76.3 U/L), normal (23.5 U/L), and low (5.9 U/L) enzymatic activities, respectively. The bias was -1.3, -1.9 and -0.6% for samples with low (17.3 U/L), normal (28.5 U/L), and high (39.6 U/L) enzymatic activities, respectively (Table 2). The results for the TAE of the in-house method are presented in Table 2.

FIGURE 3 Linearity of the in-house method for determination of ADA activity. (A) Linearity curve in all dilutions; (B) linear equations obtained with dilutions corresponding to activities ranging from 0 to 100 and 0 to 200 U/L

3.3 | Sample stability

The results of the sample stability evaluation are shown in Figure 4. The results for the pleural fluid pool with low enzymatic activity were not considered, because these demonstrated a higher analytical imprecision (CV_{WL}) than is acceptable at all analyzed temperatures. The best storage temperature for samples of pleural fluid for ADA activity analysis was found to be -20°C , at which samples were stable for up to six days (Figure 4).

3.4 | Comparison study

For the comparison study, 40 samples with enzymatic activities ranging from 21.5 to 130.5 U/L were analyzed using the in-house method and the Ebram[®] commercial test. Although a high correlation ($r = 0.998$) between the two methods was observed, the Bland-Altman analysis revealed a significant ($P < 0.05$) discordance between them. The mean bias of $-10.5\% \pm 2.85\%$ (-4.96 ± 2.95 U/L) is presented in Figure 5.

4 | DISCUSSION

A definitive diagnosis of tuberculous pleuritis is often challenging. Granulomatous inflammation can be revealed through blind or thoracoscopic pleural biopsies, but these procedures are invasive, require operator expertise, and are not widely available in developing countries. To help overcome the limitations of conventional methods for tuberculous pleuritis diagnosis, such as the lack of sensitive microbiological methods for pleural specimens and the need for invasive procedures like pleural biopsy, the search for biological markers for PF has been the objective of several studies. ADA activity shows a high accuracy for diagnosing tuberculosis when compared to other tests employed in routine investigations.¹ A recent meta-analysis reported that ADA for pleural fluid has good diagnostic accuracy with a diagnostic sensitivity and specificity of 0.94 and 0.89, respectively. It also appears to have a good negative predictive value for tuberculous pleuritis at a threshold value of around 40 U/L.^{6,17} Consequently, it is imperative to develop a reliable and affordable ADA assay that can be

FIGURE 4 ADA stability in pools of pleural fluid samples with medium and high activities under three different storage conditions for up to 14 days: room temperature (22–26°C), refrigeration (2–8°C), and freezing (-20°C). The red, green, and blue lines represent the effect of storage at room, refrigeration and freezing temperature, respectively. Continuous lines represent the results for the sample with medium activity, and intermittent lines represent the results for the sample with high activity

FIGURE 5 Mean percentage and agreement limits of differences between in-house method and Ebram® method

used to rapidly and accurately diagnose tuberculous pleuritis. In addition, the in-house method for measuring ADA activity proved to be simple, demanded little technical training, and had low operational costs because it uses affordable reagents, glassware, and equipment common to most medium-sized laboratories.

4.1 | Calibration curve of the in-house method

Using correlation/regression analysis, a correlation between the ammonium sulfate solution and the commercial calibrator would be expected. This is demonstrated by both the coefficients of correlation (r) and determination (R^2), as well as by the equation's least-square parameters (slope and intercept) of the best fit line.¹⁸ As illustrated in Figure 2, it can be assumed that the ammonium sulfate pattern is adequate for evaluating the corresponding ADA activity of the samples using the in-house method. However, it is important to highlight that the linear correlation/regression analysis measures the strength of a linear association but not the agreement between the analytical methods.¹⁸

4.2 | Characteristics and performance of the in-house method

In comparison with the linearity of the Ebram® method, which is specified by the manufacturer as up to 200.0 U/L,¹⁰ and the one from Biosystems® (150.0 U/L),⁹ the in-house method presents lower linearity. It is worth noting (Figure 3), however, that the method was linear until the dilution corresponding to 200 U/L. That said, the NCCLS EP6-A document suggests that a laboratory must determine its own goals for linearity, and these should be less than or equal to the goals for bias.¹⁹ The results from 200 U/L were therefore discarded, because the bias (19%) exceeded the minimum requirement of 10.5%

that we had previously defined according to Westgard's (2016)¹³ recommendations. It was therefore established that samples with concentrations higher than 100 U/L should be diluted and have their results corrected by the dilution factor. From 100 U/L, the in-house method is unable to provide results that are directly proportional to the ADA activity. This may lead to underestimated results and impair clinical interpretation, especially when used for disease monitoring. Nevertheless, the results are unlikely to compromise clinical diagnosis due to the distance from the upper reference limit (40 U/L).⁶

The sensitivity of the in-house method was assessed using the slope of the linear regression curve using both ammonium sulfate and commercial calibrator standards (Ebram®). Although both determinations presented similar results, the analytical sensitivity depends on the nature of the analyte and the employed detection technique.²⁰ The in-house method demonstrated the ability to distinguish activities of 0.013 U/L. While Biosystems®¹⁰ does not provide this information for its test; the analytical sensitivity specified by Ebram® for its test is lower¹⁰ than the in-house method. Clinically speaking, a high analytical sensitivity is useful for monitoring a patient's response to treatment, since sensitive methods are able to detect small variations.

There are several acceptable alternative methods for determining the LOD (the lowest activity detected by the method but not necessarily quantified), depending on the technology that is being validated,¹⁴ and these can generate quite different results. The LOD of the in-house method was lower than that of the commercial test from Biosystems®,⁹ while Ebram®¹⁰ does not provide this information for its test. An analyte with a high limit of detection could impair any interpretation of the results, because it has been suggested that in areas of low tuberculosis prevalence, the negative predictive value of ADA may be enough to exclude a patient from pleural biopsy if an ADA determination proves to be

negative. In contrast, in areas of high tuberculosis prevalence, a positive ADA determination would have a positive predictive value for tuberculosis of 88%-99%.^{2,6}

The LOD and LOQ are critical when detecting extremely small amounts of an analyte, and this is necessary to establish the state of a disease or screen for the presence of it.²¹ The results between the LOD and LOQ could still be reported, however, and possibly associated with a caveat regarding a higher uncertainty in the result.²¹

It was not possible to compare the LOQ of the in-house method with the Biosystems[®] and Ebram[®] tests¹⁰, because their manufacturers do not specify this performance characteristic.

Considering the LOD and the linearity of the in-house method, the MWR was determined (with 99% statistical confidence) between 0.2 U/L and 100.0 U/L. This means that samples with results outside this range cannot be measured precisely and accurately. Any values less than 0.2 U/L must be reported as "lower than the sensitivity of the method" or as "lower than 0.2 U/L," while values above 100 U/L must be diluted and retested before being reported.

The characteristics of the in-house method described above do not have a direct influence on errors in measurement results, or they are a consequence of them, so they do not directly endanger the decisions behind the results. The method's performance can be better evaluated using predefined quality goals as analytical specifications based on biological variation, clinical outcomes, published professional recommendations, the current state of the art, and so on.¹³

A quality specification based on biological variation is more suitable for our objectives, which are focused on the patient, since monitoring is the most demanding medical application of ADA analysis. This analytical performance goal, which is calculated based on the intra- and interindividual biological variation (CV_i and CV_g) of ADA,¹⁵ allows evaluation of the CV, bias, and TAE of the method (Table 2). It is a rigorous criterion for evaluating an analytical system, being the minimum specification for the lowest level of requirement and a great goal level for laboratory quality.¹⁵

One of the components of analytical imprecision is the within-assay CV (CV_R). The in-house method showed a CV_R slightly higher than those declared by the manufacturers of the commercial tests for medium and high levels of ADA activity (Table 2). There is no quality specification for this parameter, however. CV_R can be thought of as the imprecision inherent in the method. However, because it is a manual method, a robustness study is recommended to verify the interanalyst variability and thus confirm the repeatability of the in-house method with confidence.

The other component of analytical imprecision is the between-assay CV (CV_{WL}). For this criteria, the in-house method showed desirable performance (up to 5.9%) for high ADA activity (CV_{WL} = 5.0%) and minimum performance (up to 8.8%) for normal ADA activity (CV_{WL} = 8.8%). However, it demonstrated unacceptable imprecision for low ADA activities (CV_{WL} = 10.4%), because it did not satisfy the specification of minimum quality. The in-house method for ADA determination demonstrated lower precision when compared to the commercial tests from Ebram[®] and Biosystems[®] (Table 2).

In a sense, a method that presents high imprecision may hinder the monitoring of laboratory results over time. Borderline results may lead to difficulties when differentiating health or disease situations. Moreover, minor changes in a result could reflect a simple analytical variation in the method rather than an improvement or worsening in a patient's condition. Retesting a sample should always therefore be considered in situation when doubt or inconsistencies exist relating the clinical data. Nevertheless, we still conclude that the method's imprecision for low enzymatic activities does not compromise the clinical decision-making process, because it is far from the decision threshold (40 U/L).

In general, the in-house method demonstrates, through its very low negative bias, a small underestimation, and an optimum performance according to the quality specification based on biological variation (Table 2). Theodorsson (2012) discusses that bias measurement can be accomplished either using several approaches or using a single one.¹⁴ In our study, we sadly could not calculate the bias according to CLSI EP15 using reference materials,²² because this would cause excessive expenditure for our small laboratory. We were, however, able to calculate it using the calibration curve (with the calibrator), and this exhibited similar results (not shown).

In addition, the in-house method showed better trueness (as represented by bias) than the Ebram[®] commercial test (Table 2). Biosystems[®] does not specify the trueness of its method.

The accuracy is represented by the precision and trueness of the method (ie, the TAE). Laboratories using inaccurate methods would hardly be able to compare their results with other laboratories, making it important to perform patient monitoring using sequential results from the same laboratory.

The TAE of the in-house method varied from 8.8% to 18.4% with 95% confidence (Table 2). The TAE of the method for samples with high and medium enzymatic activities met the desirable quality specifications (up to 16.7%). For low enzymatic activity, the method showed minimum performance. The inaccuracy inherent in the method therefore did not exceed the biological variation of ADA, thus assuring analytical validation and the method's ability to report reliable and fit-for-purpose results.

4.3 | Evaluation of the stability of the pleural fluid samples

The sample stability study evaluated the variation in the analyte under different storage conditions over time. This is important for determining the best storage temperature and the maximum time a sample can be stored for examination or reanalysis when necessary, because any degradation in the analyte or matrix during storage may affect the accuracy of the results.

The maximum allowed variation in the results for stored samples was presumed to be the acceptable random error, which is up to 8.8% according to quality specifications based on biological variation. This ensures that any percentage loss above this value will be related to sample instability rather than any imprecision in the analytical system.

The commercial Ebram® test specifies stability information only for serum and plasma, which is up to seven days in the refrigerator (2–8°C) or one month when frozen (–20°C).¹⁰ Similar to our results, Biosystems® reports a seven-day stability for both serum and pleural fluid under refrigeration (2–8°C).⁹ The study of the sample stability is complementary to the validation process, but it is an important factor in the pre-analytical phase that needs standardization in order to avoid errors that could affect a clinical interpretation.²³

4.4 | Method comparison study

Analytical validation studies typically include a method comparison experiment with the purpose of identifying the inaccuracy of a “new” method relative to an “established” method. It is particularly relevant when demonstrating that a new method is a satisfactory substitution for an established one.

A good correlation was observed between the commercial test and the in-house method. But a high correlation or association does not automatically imply a good agreement between the two methods. The calculated slope and intercept suggest that the ADA activity obtained with our in-house method is lower than that obtained with the commercial test. This negative bias of the method was previously observed elsewhere.

A Bland-Altman analysis is preferable to a correlation test, because it shows the interval of the agreement. However, it does not specify whether those limits are acceptable or not. Acceptable limits must be defined a priori based on clinical necessity, biological considerations, or other goals.²⁴ In our comparison study, the statistical analysis shows that even though the methods presented results that correlate, they do not agree. However, despite the fact that the results of the two methods are significantly different on average, the difference (–10.5%) is acceptable according to quality specifications based on biological variation. This suggests that the in-house method is suitable for its intended use and allows proper decisions to be made with a good degree of confidence.

Our study has some limitations in terms of its design. First, the studies were performed individually due to the limited number of PF samples and their volume and stability. Second, there are other parameters that could have been analyzed, such as robustness, different matrices, reference interval, reagent stability, and uncertainty measurement. Further studies are also needed to characterize the pre-analytical, endogenous, and exogenous interferences in the in-house method for ADA activity measurement. In addition, we have not found a free guide with a complete description of all the needed methodologies. As a small laboratory, we experience difficulties when trying to access internationally accepted standard analysis methods, such as CLSI.

Careful assessment of potential analytical interferences should be undertaken through dialogue between clinicians and laboratory technicians when any discordances between clinical and laboratory data are detected. Although this ADA test has high diagnostic sensitivity and specificity, the ADA test result should be

interpreted based on a combination of clinical and laboratory findings. Further studies regarding the measurement of screening biomarkers and rapid tuberculosis diagnosis are therefore suggested.

The findings demonstrate the potential of the in-house method, which shows reliable analytical performance and accurate results that are consistent with commercial tests. This makes it suitable for use in routine diagnostics. In particular, it would allow routine tuberculous pleuritis diagnosis with an MWR in accordance with the threshold value of around 40 U/L, as suggested by clinical practice guidelines.

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