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COMPARISON OF TWO ENZYMATIC IMMUNOASSAYS, HIGH RESOLUTION MASS SPECTROMETRY METHOD AND RADIOIMMUNOASSAY FOR THE QUANTIFICATION OF HUMAN PLASMA HISTAMINE

Running head: Method comparison for human histamine quantification

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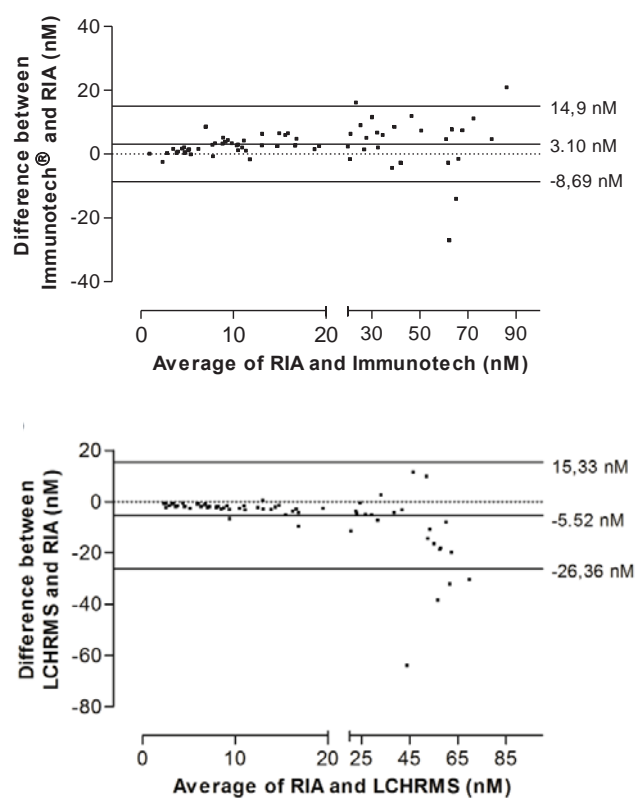
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Graphical abstract



Highlights

- LCHRMS and Immunotech[®] EIA both displayed high correlation with RIA
- LCHRMS displayed high sensitivity and specificity
- 7.88nM and 14.39nM was determined as new cut-offs for LCHRMS and Immunotech[®] EIA

ABSTRACT

Histamine (HA) is one of the main immediate mediators involved in allergic reactions. HA plasma concentration is well correlated with the severity of vascular and respiratory signs of anaphylaxis. Consequently, plasma quantification of HA is useful to confirm the diagnosis of anaphylaxis. Currently, radioimmunoassay (RIA) is the gold standard method to quantify HA due to its high sensitivity, but it is time consuming, implicates specific formations and cautions for technicians, and produces hazardous radioactive wastes. The aim of this study was to compare two enzymatic immunoassays (EIA) and one in-house liquid chromatography high-resolution mass spectrometry method (LC-HRMS) with the gold standard method for HA quantification in plasma samples of patients suspected of anaphylaxis reactions. Ninety-two plasma samples were tested with the 4 methods (RIA, 2 EIA and LC-HRMS) for HA quantification. Fifty-eight samples displayed HA concentrations above the positive cut-off of 10 nM evaluated by RIA, including 18 highly positive samples (> 100 nM). Our results showed that Immunotech[®] EIA and LC-HRMS concentrations were highly correlated with RIA values, in particular for samples with a HA concentration around the positive cut-off. In our hands, plasma concentrations obtained with the Demeditec Diagnostics[®] EIA correlated less with results obtained by RIA, and an underestimation of plasma HA levels led to a lack of sensitivity. In conclusion, this study demonstrates that Immunotech[®] EIA and LC-HRMS method could be used instead of RIA to assess plasma HA in human diagnostic use.

ABBREVIATIONS

EIA: enzymatic immunoassay

HA: histamine

LC-HRMS: liquid chromatography high resolution mass spectrometry

RIA: radioimmunoassay

Keywords: enzymatic immunoassay, histamine, mass spectrometry, radioimmunoassay

1. INTRODUCTION

Anaphylaxis is a potentially lethal systemic type I hypersensitivity reaction [1]. This life-threatening emergency can lead very quickly to hypovolemia shock and cardiorespiratory arrest. In western countries, anaphylaxis incidence is estimated to be comprised between 4 and 50/100000 inhab/year with a prevalence of 0.05–2%. Emergency admission registers highlight a two fold increase of anaphylaxis prevalence during the last decades [2].

After a first exposure to an allergen leading to high-affinity IgE synthesis by long-lived plasma cells, these IgE are predominantly found linked to the high-affinity IgE receptor (FcεRI) at the cell surface of mast cells and basophils. Following a second exposure to the same allergen, the cross-linking of the FcεRI leads to cell activation and the release within a few minutes of a huge amount of histamine (HA) contained in cell granules (10^{-5} to 10^{-3} mol/L) [3, 4], as well as other preformed proinflammatory mediators [5]. HA or 2-(1H-imidazol-4-yl) ethanamine is synthesized from L-histidine in the Golgi apparatus by the histidine decarboxylase [6]. Several cell types can synthesize HA in humans [7], but mast cells and basophils distinguish themselves by their ability to store HA in their granules, enabling a huge acute release of HA upon IgE cross-linking.

The anaphylaxis diagnosis is usually retained on a body of evidence. Suspicion is made primarily on clinical signs [8-10]. Currently, two biological parameters are commonly used to strengthen the diagnosis of anaphylaxis: serum tryptase and plasma HA. HA quantification is useful to confirm that clinical signs result from the degranulation of mast cells and/or basophils [11]. Physiologically, basal plasma HA amount is very low (<10 nM) [8] but can increase very quickly and exponentially after mast cell or basophil degranulation. Nevertheless, the HA protein half-life is extremely short [12]. Consequently, strict procedures of sampling for HA quantification are required: blood collection must be conducted within 15-60min after the beginning of the symptoms and plasma isolation has to be performed very

quickly before conservation [13]. The production of highly specific monoclonal antibodies targeting HA is extremely challenging due to the nature of this small biogenic amine ($M=111\text{g.mol}^{-1}$). The former quantification assays were based on the covalently binding of HA to a carrier protein on imidazole core or on NH_2 end; nevertheless this chemical process was difficult to apply on plasma samples. Currently, classical HA quantification assays used for *in vitro* diagnosis (IVD) and based either on radioimmunoassay (RIA) [14] or enzymatic immunoassay (EIA) methods include an acylation step with NOH succinimide ester succinyl glycineamide, which turns HA into a bigger and more stable molecule that becomes easily detectable. Acylated plasma HA competes with exogenous acylated HA conjugated with a radioelement or an enzyme to bind a highly specific monoclonal antibody [15]. The current gold standard for HA quantification is RIA, it has the advantage of being highly sensitive [14], but requires the use of radioactive reagents implying the formation and the follow up of the technicians, as well as the dangerous and expensive waste management. More recently, two EIA kits for IVD use, both avoiding the use of radioelements, have been commercialized by Immunotech® and Demeditec Diagnostics®. Finally, some methods using mass spectrometry have been developed to quantify plasma HA [16, 17]. In particular, our team developed an in-house plasma HA quantification assay for diagnostic use based on high Resolution Accurate Mass LC-MS (LC-HRMS) technology [18]. The LC-HRMS method is known to be highly sensitive and specific, averts radioactivity use, and avoids the potential detection of the metabolites of the targeted protein.

In this study, HA concentration was assessed in human plasma collected from patients with suspected anaphylactic reaction using three different methods: the gold standard RIA, the two commercialized EIA kits, and the in-house LC-HRMS-based method. This comparison reveals that Immunotech® EIA and LC-HRMS are both sensitive enough methods to be considered as relevant non-radioactive alternative methods to RIA.

2. MATERIALS AND METHODS

2.1 Plasma samples

Plasmas collected between February 2010 and February 2014 in routine clinical practice by laboratories of Angers and Rennes University Hospitals from patients following a suspected anaphylaxis reaction were tested for HA quantification. The Ethical Review Board in Angers approved this study (2015/40). Peripheral blood was collected by venipuncture in EDTA tubes, and plasma was separated by centrifugation at 3000g for 10min. Thereafter, plasma was aspirated gently while respecting a safety margin of 1cm from the buffy coat in order to avoid contamination by white blood cells, aliquoted and immediately frozen at -20°C.

2.2 Radioimmunoassay

HA quantification by RIA was performed following manufacturer's instructions (Immunotech, Marseille, France). Briefly, immediately after thawing, HA contained in plasma samples was acylated. Then, plasma samples were incubated overnight within monoclonal antibody (mAb)-coated tubes in presence of a ^{125}I -labeled acylated HA internal tracer. Following incubation, the contents of the tubes were aspirated and bound radioactivity was measured using a Cobra 2 auto gamma counter (Packard instrument, Meriden, CT, USA). Limits of quantification given by the manufacturer were 0.2-100nM. The limit for pathological values is 10nM.

2.3 Enzymatic immunoassays

HA quantification by EIA was performed following manufacturers' recommendations (Immunotech® and Demeditec Diagnostics®, Kiel, Germany). For both kits, samples were manipulated in polypropylene tubes and tested in duplicate.

For Immunotech® EIA, HA was acylated by a first vortex step with an acylation reagent. Acylated control, standard and plasma solutions were further incubated in mAb-coated wells in presence of alkaline phosphatase acylated HA for 2 hours at 2-8°C under shaking (350rpm). After incubation, wells were rinsed with a microtiter plate washer in order to remove non-bound components. The remaining enzymatic activity was then measured at 405nm after the addition of a chromogenic substrate. A quadratic polynomial equation was used to define the standard curve; the correlation coefficient r was always >0.993 [0.987-0.999], and the median control values were 5.561 [95% CI 5.459-5.660] nM for theoretical value between 5.00 and 7.40nM. Standard curve included dilutions from 1.00 to 100nM of HA, as recommended. Limits of quantification given by the manufacturer were 0.50-100nM.

For Demeditec Diagnostics® EIA, HA was acylated by incubation with an acylating reagent for 45min at room temperature (RT) on a shaker (600rpm) and further competed with plastic-coated HA for a precise amount of HA antibody binding sites added into the well. Equilibrium of the system was reached after 3 hours at RT (20-25°C) on a shaker (approximately 600rpm). Then, free antibody and plasma HA-antibody complexes were removed by washing. The antibodies conjugated with plastic-coated HA were detected by a peroxidase-conjugated anti-rabbit IgG using tetramethylbenzidine as substrate. The reaction was monitored at 450nm. A quadratic polynomial equation was used to define the standard curve, included standard concentrations between 4.5 and 450nM of HA, as recommended. The correlation coefficient r was always >0.99 [0.992-1.000]. The median control values were 26.1nM [22.0-33.7] and 80.4nM [60.6-102.5] for theoretical values of 30.0 and 100.0nM, respectively. The manufacturer gave no target range of concentration for the controls. Limits of quantification given by the manufacturer were 6.6-76.0nM.

2.4 High Resolution Accurate Mass LC-MS technology

A liquid chromatography coupled with an ultra-high resolution and accurate mass instrument was used to determine HA concentration. Briefly, 100 μ L of plasma was added to 250 μ L of de-ionized water and 100 μ L of internal standard Histamine-d4 (HAd4) (C/D/N isotopes, Quebec, Canada). Sulfosalicylic acid (50 μ L at 20% v/v) (Sigma-Aldrich, Saint-Louis, MO, USA) was added for protein precipitation. After centrifugation at 3000g for 10min at 4°C, 200 μ L of supernatant were transferred to an auto-sample vial before analysis. Separation of the analytes was achieved on a C18 Accucore column (100 mm x 2.1, 2.6 μ m) (Thermo Scientific, San José, CA, USA) using a gradient run with solvent A (nonafluoropentanoic acid 3mM) and solvent B (acetonitrile-formic acid 0.1:100, v/v) formic acid). The analytes were detected using a Q ExactiveTM (Thermo Scientific) mass spectrometer operating in positive electrospray ionisation. In full scan mode, the exact mass of each protonated species (112.0869 for HA; 116.1120 for HAd4) was extracted for quantification; one ion transition was monitored per compound for quantification in fragmentation mode (HA: 112.0869>95.0607 m/z; and HAd4: 116.1120> 99.0855 m/z). The calibration curve for HA in plasma was linear from 1 to 100nM.

2.5 Statistical analysis

Sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) of EIA and LC-HRMS were calculated and compared to RIA. A threshold of 10nM was used, because 10nM is considered the classical admitted limit for pathological values [8], and corresponds to the cut-offs given by manufacturers for RIA and EIA kits. Then, the analyses were restricted to the samples with HA measures comprised within the quantification limits of the two methods. HA concentrations measured by LC-HRMS, and Immunotech[®] and Demeditec Diagnostics[®] EIA were compared using Pearson correlation

coefficient with concentrations obtained with RIA. Bland-Altman tests were performed to evaluate the agreement of each method *versus* RIA. Bias and 95th limits of agreement (LOA, average difference \pm 1.96SD of the difference) were calculated. Samples with a RIA-measured HA concentration comprised between 1 and 20nM were further specifically investigated, because this range of concentrations could be delicate to interpret by the clinicians, and must be interpreted together with the severity of the symptoms and the results of additional biological tests, in order to conclude to anaphylaxis. Finally, receiver operating characteristic (ROC) curves were used to determine the new cut-off values for EIA Immunotech[®] and LC-HRMS methods; the optimal cut-off point being the threshold that maximizes the sum of the sensitivity and the specificity (Youden's index). GraphPad Prism 5.0 (GraphPad software, San Diego, CA, USA), Excel and Stata (College Station, TX, USA) softwares were used.

3. RESULTS AND DISCUSSION

3.1 Cohort recruitment and HA quantification by RIA

This study aimed to compare HA concentrations of human plasma samples from patients with suspected anaphylactic reaction quantified with several assays for *in vitro* diagnostic use. HA measurement for 34 samples (37%) was quantified below the threshold of 10nM by RIA, and was consequently considered negative in routine clinical practice. One of these 34 samples was quantified below 0.2nM corresponding to the RIA lower limit of detection. Fifty-eight samples (63%) were considered positive with HA concentration measured by RIA above 10nM, including 18 samples with a concentration above the RIA upper limit of quantification of 100nM. Globally, HA concentrations of our cohort of 92 samples were well distributed over the quantification range of the RIA. The RIA method remains the historical gold standard for HA quantification in biologic fluids since several decades. Despite its high

sensitivity (analytical sensitivity: 0.2nM), this radioactive method displays several disadvantages, including the exposition risk of technicians to ^{125}I and the management of radioactive wastes. Furthermore, the use of the radioactive reagents restricted to a specific window of radioactive decay leads to peculiar ordering of reagents, increases the delay of reagent shipment, and consequently expands the waiting time of results for the patient. We selected 3 alternative assays using 2 different methods: Immunotech[®] EIA, Demeditec Diagnostics[®] EIA and LC-HRMS (Table S1) [18, 19]. HA concentration values obtained by RIA were compared with the HA concentrations obtained with each of the other methods, based on a list of samples presenting a HA concentration in the detection limits of both tested methods.

3.2 Comparison of Immunotech[®] EIA to RIA

HA concentration of the 92 tested samples was evaluated with RIA and Immunotech[®] EIA. Considering all samples, sensitivity of the Immunotech[®] EIA method was 100% with no false negative, and the specificity was 59% (table 1). Indeed, 14 false positive results were obtained with the Immunotech[®] EIA method with HA quantification values comprised between 11 and 16nM. Comparing HA concentrations measured with the 2 methods, a maximal difference of 8.6nM for 2 samples was obtained. Finally, the positive and negative predictive values were 81% and 100%, respectively (table 1).

Among the 92 samples from our cohort, 91 were within the detection limits of RIA. Among the 91 samples, 72 (79%) were between 0.5 and 100nM, the detection limits of Immunotech[®] EIA. We therefore decided to only compare these 72 selected samples, and calculated a Pearson's correlation coefficient r of 0.96 (95% CI 0.94-0.98; $p < 0.0001$) for this sample set (figure 1A). The Bland-Altman plot comparing Immunotech[®] EIA to RIA values showed an

estimated bias of 3.10nM (SD: 6.02nM; 95% limits of agreement: -8.69 - 14.90nM) (figure 1B).

Because HA concentrations between 1 and 20nM could be arduous to interpret for clinicians [20], a focus was made on samples displaying concentrations measured by RIA comprised in this range (n=47), and a Pearson's correlation coefficient of 0.88 (95% CI 0.80-0.93; $p < 0.0001$) was calculated (Figure 1C). The subsequent Bland-Altman plot showed an estimated bias of +2.97nM (SD: 3.08nM; 95% limits of agreement: -3.07 - 9.01nM) (table 2).

Globally, Immunotech® EIA and RIA values were well correlated even if a slight overestimation of HA concentration using Immunotech® EIA was observed. Of note, Immunotech® EIA kit has been developed by the same manufacturer and shares with the RIA kit most of the reagents. Essentially only the revelation step (^{125}I -labeled histamine tracer *versus* histamine-phosphatase alkaline conjugate) is different. Interestingly, EIA methods avoid staff exposition to radiation hazards, are easy to perform, and do not require specifically trained staff, unlike RIA methods. Moreover, equipment needed is inexpensive and widely available.

In conclusion, besides a slight overestimation of HA concentrations, all these observations support the hypothesis that EIA can be a favorable alternative to RIA, as described previously for other molecules like progesterone, cortisol, or infliximab [21-23].

3.3 Comparison of Demeditec Diagnostics® EIA to RIA

For the 92 plasma samples tested, no false positive were found resulted in 100% specificity for Demeditec Diagnostics EIA *versus* RIA. Nevertheless, 20 false negative values were observed leading to a sensitivity of 66% (table 1).

Among the 91 samples within the detection limits of RIA, only 23 plasma samples (25%) were between 6.66 and 76nM, the limits of detection of Demeditec Diagnostics® EIA, and

were further compared to RIA. The Pearson's correlation coefficient r calculated for the 23 samples was 0.45 (95% CI: 0.05-0.73; $p=0.0311$) (figure 2A). As shown in the Bland-Altman plot, HA quantification was underestimated with Demeditec Diagnostics[®] EIA with a mean difference of -17.85nM (SD: 20.16nM; 95% limits of agreement: -57.36 - 21.66nM) (figure 2B). Finally, only 4 samples quantified between 1 and 20nM by RIA were in the limits of quantification of Demeditec Diagnostics[®] EIA kit, so it was not judicious to specifically focus on these samples.

In conclusion, in our hands, the use of Demeditec Diagnostics[®] EIA resulted in an important proportion of false negative samples, which could be an obstacle for the diagnosis of anaphylaxis. A poor correlation with RIA values was obtained, despite excellent correlation coefficients obtained for the standard curves built with 5 standard dilutions ($r>0.99$). Comparing Demeditec Diagnostics[®] EIA, Immunotech[®] EIA and RIA protocols, we noticed a longer acylation step for Demeditec Diagnostics[®] EIA (45min *versus* an immediate acylation by vortexing for the 2 other assays). This could explain the important underestimation of HA quantification for the Demeditec Diagnostics[®] EIA method, because it is well known that HA remains stable only after acylation.

3.4 Comparison of LC-HRMS to RIA

For the 92 plasma samples tested, LC-HRMS sensitivity was 93% with 4 false negatives, and specificity was 100% with no false positive. The PPV and NPV were 100% and 89%, respectively (table 1).

Seventy-one of the 92 tested plasma samples showed HA concentrations comprised within the limits of quantification of the 2 methods. The Pearson's correlation coefficient r calculated on the 71 samples was 0.90 (95% CI 0.84-0.94; $p<0.0001$) (figure 3A). Bland-Altman plot showed an underestimation of the LC-HRMS method (bias: -5.52nM; SD: 10.64nM; 95%

limits of agreement: -26.36 - 15.33nM) (figure 3B). When considering the 46 samples with a HA quantification comprised between 1 and 20nM by RIA, LC-HRMS results remained highly correlated to those obtained using RIA (Pearson's correlation coefficient $r = 0.97$; 95% CI: 0.95-0.98; $p < 0.0001$) (figure 3C), and a mean difference of -1.94nM (SD: 1.26nM; 95% limits of agreement: -4.41 - 0.53nM) was obtained for the Bland-Altman analysis (table 2). At this same range, Immunotech® EIA was weakly correlated with a correlation coefficient of 0.88, was still very sensitive, but display many false positive results that could lead to overdiagnosis (Figure 1C).

In conclusion, besides a slight underestimation of HA concentrations measured by LC-HRMS, LC-HRMS gave particularly encouraging results, as a good correlation with RIA was found [18].

3.5 Comparison of the 3 tested assays to RIA

The 4 tested assays display similar characteristics with respect to their lower limit of quantification and their quantification range, except for the Demeditec Diagnostics® EIA which display a lower limit of quantification (LLOQ) of 6.66nM, close to the positive threshold of 10nM. Our results highlight a very high sensitivity for Immunotech® EIA and LC-HRMS (100% and 93%, respectively), in contrast to Demeditec Diagnostics® EIA (66%). This latter assay showed 20 false negative among the 58 samples considered as positive with a threshold of 10nM. Among these 20 false negative results, RIA quantified 3 of them as having a concentration higher than 70nM. Demeditec Diagnostics® EIA and LC-HRMS showed a perfect specificity (1.00 for both), compared to RIA. A high number of false positives were found for Immunotech® EIA (14 out of 72 positive samples), but the mean HA concentration of these samples was 11.91 \pm 1.40nM, which represent concentrations close to the positive threshold of 10nM.

It is well known that HA is very quickly metabolized *in vivo* by the histamine N-methyltransferase (HNMT) and the diamine oxydase (DAO) with a short half-life estimated at 102 seconds (17). A degradation of HA for a specific test can be rule out, as samples for each assay were aliquoted and frozen simultaneously. Thawing procedure in ice was also the same for the 4 assays. According to the manufacturers' data, cross-reactivity with the metabolite methyl histamine is estimated at 0.1% in Demeditec Diagnostics[®] EIA, and cross-reactivity to acylated methyl histamine is estimated at 0.027%, and 0.069% in Immunotech[®] EIA and RIA, respectively. LC-HRMS is the only assay that can distinguish methyl histamine from HA [16-18]. This could explain the trend of LC-HRMS to slightly underestimate plasma HA concentrations in comparison to RIA.

3.6 New cut-offs for Immunotech[®] EIA and LC-HRMS assays

At the sight of statistical results obtained with Immunotech EIA and LC-HRMS method, we decided to optimize the sensitivity and specificity of these 2 assays by determining new cut-offs. For this purpose, Ninety-two plasma samples have been used to build ROC curves in order to define new cut-offs for Immunotech EIA[®] kit and LC-HRMS method. Using the cut-off value of 10nM of HA after quantification with RIA, 58 plasma samples were classified as positive ($\geq 10\text{nM}$) and 34 as negative ($< 10\text{nM}$). Considering HA concentrations obtained for Immunotech EIA[®], the area under the curve (AUC) was 0.990, and with a cut-off value of 14.39nM (figure 4A), a sensitivity of 96.55% and a specificity of 97.06% was calculated. For LC-HRMS, the calculated AUC was 0.994 and the cut-off value was defined at 7.88nM with a sensitivity of 97.06% and a specificity of 98.28% (figure 4B). Using these new cut-offs and for the 92 tested samples, this allows minimizing the number of false positive samples from 14 to 1 for Immunotech EIA, and reducing the number of false negative from 4 to 1 for LC-HRMS (table 3).

4. CONCLUSION

Our results demonstrate that LC-HRMS and Immunotech® EIA could be good alternatives to RIA for HA quantification in medical laboratories, after optimization of the sensitivity and the specificity of the two assays using new cut-offs (7.88 and 14.39nM for LC-HRMS and Immunotech® EIA, respectively). These two methods have the advantage to avoid the use of radioactivity with all its constraints. However, rare are the laboratories possessing a high-resolution mass spectrometer due to the price of the instruments, and this could limit the development of HA quantification with LC-HRMS.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1: Correlation and Bland-Altman analyses for Immunotech® EIA *versus* RIA for the quantification of histamine concentration in plasma samples. (A) Scatter plot of HA concentrations of the 72 plasma samples measured with Immunotech® EIA and RIA, and associated Pearson's correlation coefficient r . (B) Bland-Altman plot for Immunotech® EIA *versus* RIA. Y axis: $100 \times (\text{EIA} - \text{RIA}) / \text{average}$; X axis: $\text{average} = (\text{EIA} + \text{RIA})/2$. (C) Scatter plot of HA concentrations of the 47 plasma samples presenting a concentration between 1 and 20 nM by RIA, measured with Immunotech® EIA and RIA, and associated Pearson's correlation coefficient r .

Figure 2: Correlation and Bland-Altman analyses for Demeditec Diagnostics® EIA *versus* RIA for the quantification of histamine concentration in plasma samples. (A) Scatter plot of HA concentrations of the 23 plasma samples measured with Demeditec EIA and RIA, and associated Pearson's correlation coefficient r . (B) Bland-Altman plot for Demeditec Diagnostics® EIA *versus* RIA. Y axis: $100 \times (\text{EIA} - \text{RIA}) / \text{average}$; X axis: $\text{average} = (\text{EIA} + \text{RIA})/2$.

Figure 3: Correlation and Bland-Altman analyses for LC-HRMS *versus* RIA for the quantification of histamine concentration in plasma samples. (A) Scatter plot of HA concentrations of the 71 plasma samples measured with LC-HRMS and RIA, and associated Pearson's correlation coefficient r . (B) Bland-Altman plot for LC-HRMS *versus* RIA. Y axis: $100 \times (\text{LC-HRMS} - \text{RIA}) / \text{average}$; X axis: $\text{average} = (\text{EIA} + \text{RIA})/2$. (C) Scatter plot of HA concentrations of the 46 plasma samples presenting a concentration between 1 and 20 nM by RIA, measured with LC-HRMS and RIA, and associated Pearson's correlation coefficient r .

Figure 4: Cut-off determination for the Immunotech® EIA and LC-HRMS method. Fifty-eight positive and 34 negative samples using a cut-off of 10 nM of histamine measured by RIA were used to build the ROC curves. The black arrows point the best cut-off values: 14.39 nM and 7.88 nM were defined as the best cut-off values for Immunotech® EIA (A) and LC-HRMS (B), respectively.

Figure 1

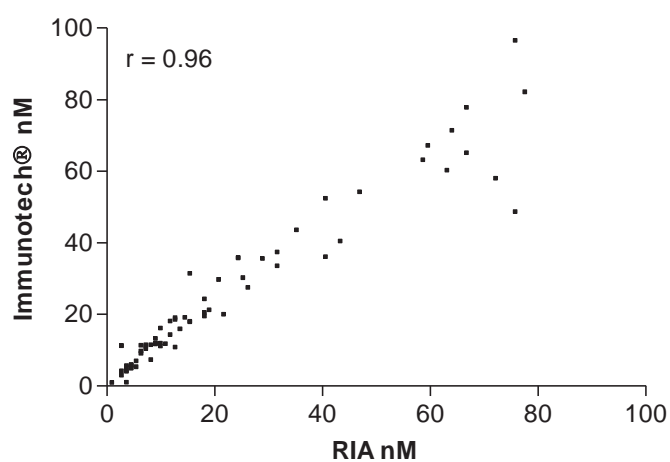
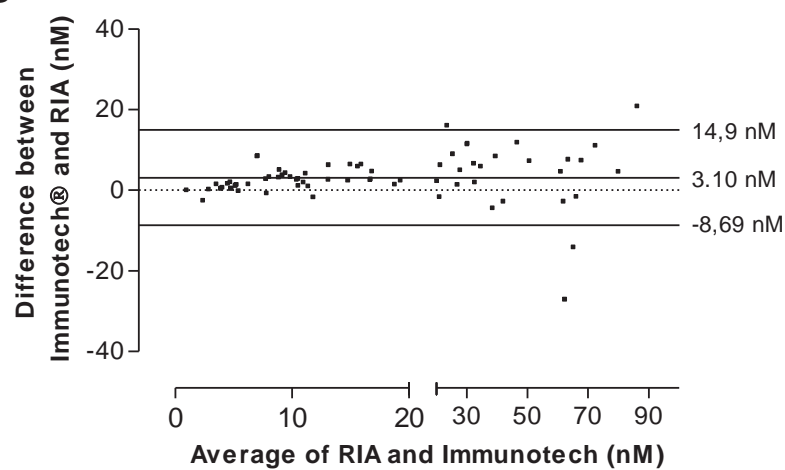
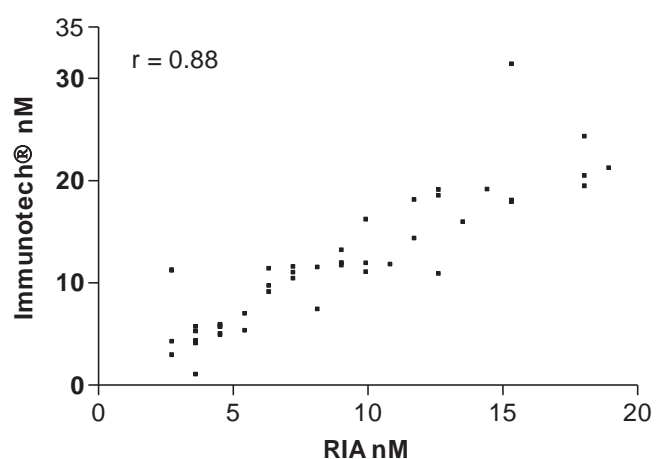
A**B****C**

Figure 2

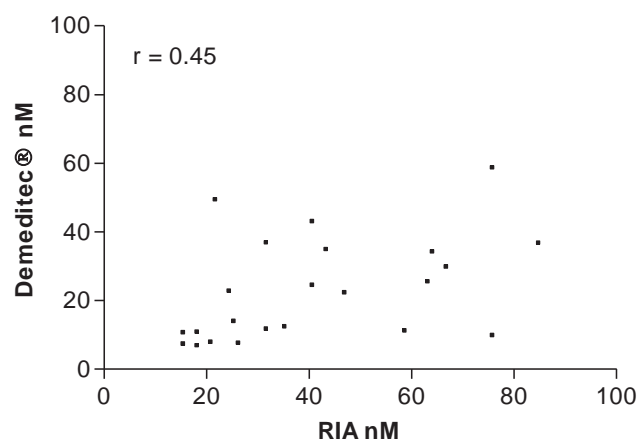
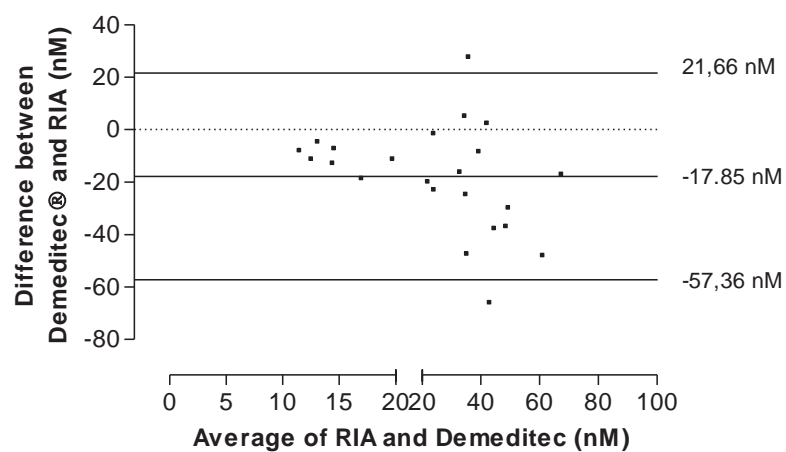
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Figure 3

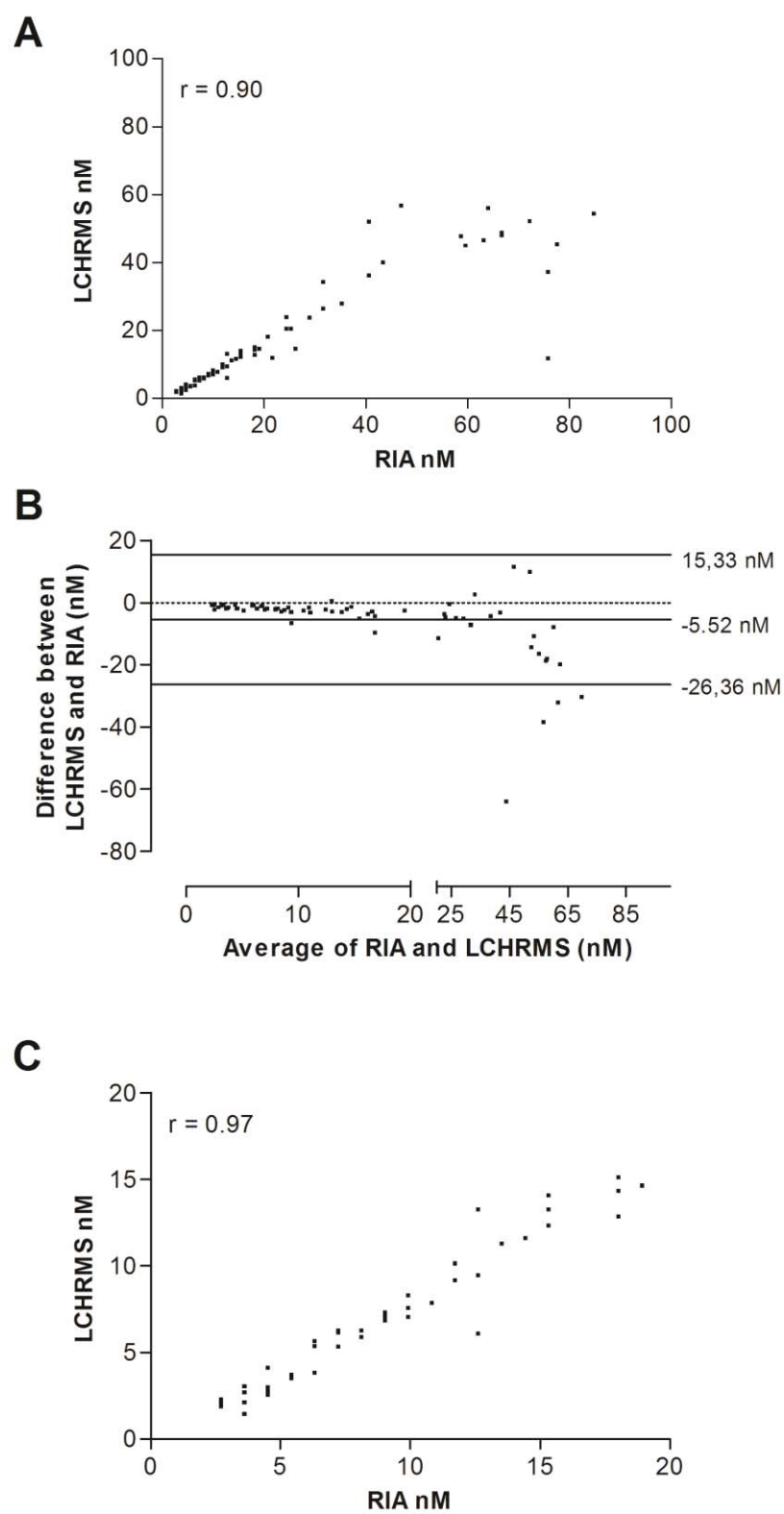


Figure 4

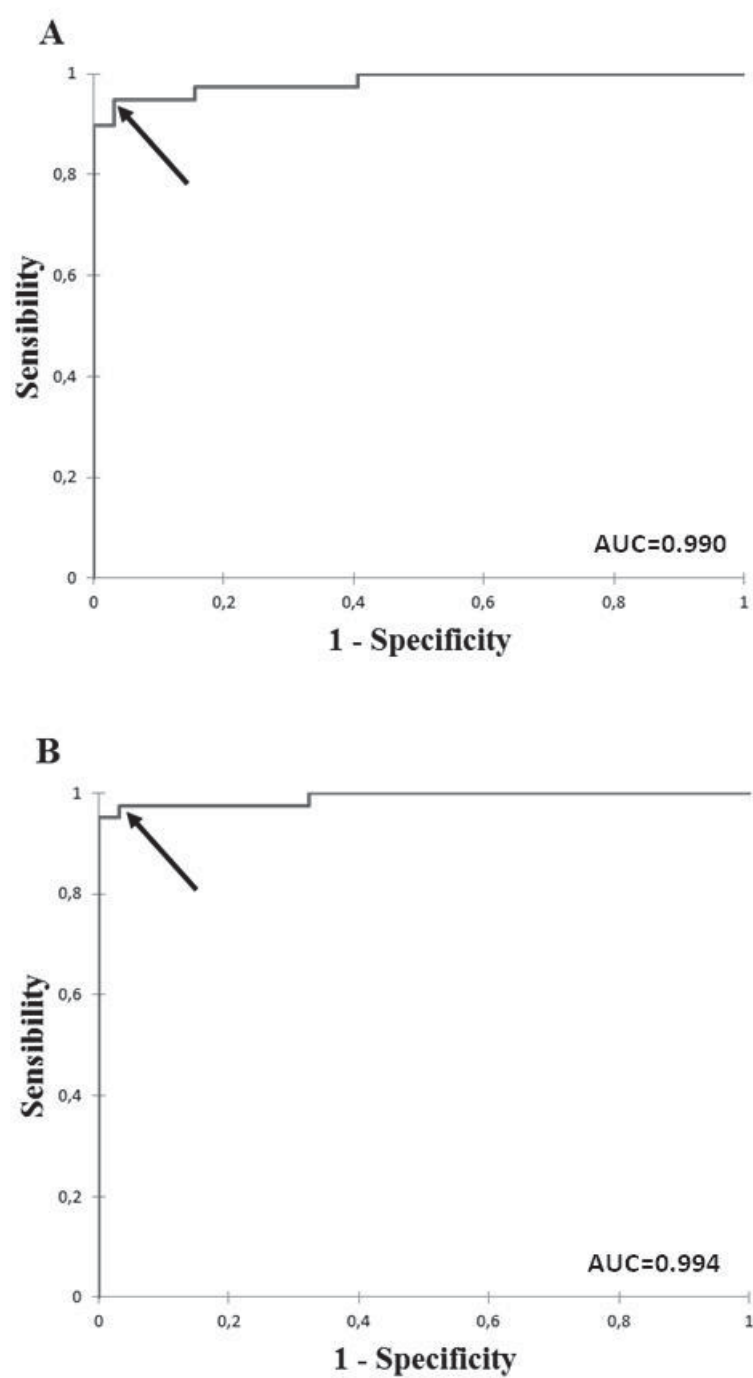


Table 1: comparison of HA quantification in plasma samples analyzed by Immunotech® and Demeditec Diagnostics® EIA, LC-HRMS and RIA. (PPV: Positive Predictive Value, NPV: Negative Predictive Value)

		RIA		Total	
		<10nM	≥10nM		
Immunotech® EIA	<10nM	20 True negative	0 False negative	20	NPV = 100%
	≥10nM	14 False positive	58 True positive	72	PPV = 81%
Total		34	58	92	
		Sp = 59%	Se = 100%		
		RIA		Total	
		<10nM	≥10nM		
Demeditec Diagnostics® EIA	<10nM	34 True negative	20 False negative	54	NPV = 63%
	≥10nM	0 False positive	38 True positive	38	PPV = 100%
Total		34	58	92	
		Sp = 100%	Se = 66%		
		RIA		Total	
		<10nM	≥10nM		
LC-HRMS	<10nM	34 True negative	4 False negative	38	NPV = 89%
	≥10nM	0 False positive	54 True positive	54	PPV = 100%
Total		34	58	92	
		Sp = 100%	Se = 93%		

Table 2: Bias \pm SD and 95% limits of agreement for HA quantification in plasma samples within the limits of detection of the methods, and focused on samples with a HA concentration measured by RIA comprised between 1 and 20nM.

	Bias \pm SD	95% limits of agreement
Immunotech[®] EIA <i>vs</i> RIA	3.10 \pm 6.02 nM	-8.69 - 14.90 nM
Immunotech[®] EIA <i>vs</i> RIA (1-20nM)	2.97 \pm 3.08 nM	-3.07 - 9.01 nM
Demeditec[®] EIA <i>vs</i> RIA	-17.85 \pm 20.16 nM	-57.36 - 21.66 nM
Demeditec[®] EIA <i>vs</i> RIA (1-20nM)		
LC-HRMS <i>vs</i> RIA	-5.52 \pm 10.64 nM	-26.36 - 15.33 nM
LC-HRMS <i>vs</i> RIA (1-20nM)	-1.94 \pm 1.26 nM	-4.41 - 0.53 nM

Table 3: comparison of HA concentrations in plasma samples quantified by Immunotech® EIA, LC-HRMS and RIA using optimized cut-offs. (PPV: Positive Predictive Value, NPV: Negative Predictive Value)

		RIA			
		<10nM	≥10nM	Total	
Immunotech® EIA	<14.39 nM	33 True negative	2 False negative	35	NPV = 97.06%
	≥14.39 nM	1 False positive	56 True positive	57	PPV = 98.25%
	Total	34	58	92	
		Sp = 97.06%	Se = 96.55%		
		RIA			
		<10nM	≥10nM	Total	
LC-HRMS	<7.88 nM	33 True negative	1 False negative	34	NPV = 97.06%
	≥7.88 nM	1 False positive	57 True positive	58	PPV = 98.28%
	Total	34	58	92	
		Sp = 98.28%	Se = 97.06%		