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RESEARCH PAPER

An enzyme-linked immuno-mass spectrometric assay with the substrate adenosine monophosphate

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Abstract An enzyme-linked immuno-mass spectrometric assay (ELIMSA) with the specific detection probe streptavidin conjugated to alkaline phosphatase catalyzed the production of adenosine from the substrate adenosine monophosphate (AMP) for sensitive quantification of prostate-specific antigen (PSA) by mass spectrometry. Adenosine ionized efficiently and was measured to the femtomole range by dilution and direct analysis with micro-liquid chromatography, electrospray ionization, and mass spectrometry (LC-ESI-MS). The LC-ESI-MS assay for adenosine production was shown to be linear and accurate using internal $^{13}C^{15}N$ adenosine isotope dilution, internal $^{13}C^{15}N$ adenosine one-point calibration, and external adenosine standard curves with close agreement. The detection limits of LC-ESI-MS for alkaline phosphatase–streptavidin (AP-SA, ~190,000 Da) was tested by injecting 0.1 μl of a 1 pg/ml solution, i.e., 100 attograms or 526 yoctomole (5.26E−22) of the alkaline-phosphatase labeled probe on column (about 315 AP-SA molecules). The ELIMSA for PSA was linear and showed strong signals across the picogram per milliliter range and could robustly detect PSA from all of the prostatectomy patients and all of the female plasma samples that ranged as low as 70 pg/ml with strong signals well separated from the background and well within the limit of quantification of the AP-SA probe. The results of the ELIMSA assay for PSA are normal and homogenous when independently replicated with a fresh standard over multiple days, and intra and inter diem assay variation was less than 10 % of the mean. In a blind comparison, ELIMSA showed excellent agreement with, but was more sensitive than, the present gold standard commercial fluorescent ELISA, or ECL-based detection, of PSA from normal and prostatectomy samples, respectively.

Keywords ELISA · Liquid chromatography · Electrospray · Mass spectrometry · Adenosine monophosphate · Alkaline phosphatase · ELIMSA

Abbreviations

AMP	Adenosine monophosphate
AP-SA	Alkaline phosphatase–streptavidin
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
ELIMSA	Enzyme-linked mass spectrometric assay
LC-ESI-MS	Liquid chromatography electrospray ionization and mass spectrometry
PSA	Prostate-specific antigen
SIM	Single ion monitoring

Introduction

ELISA stands for enzyme-linked immunosorbent assay that is a commonly used method to measure nanogram, or about picomole, amounts of proteins from complex mixtures [1, 2].

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ELISA may achieve great selectivity since two independent antibodies need to bind to the analyte and each acts as a specific filter. ELISA is typically performed in 96-well dishes that may be read by colorimetric, fluorescent, or ECL-based methods and may reach about the nanogram range. Alkaline phosphatase (AP) is a near perfect enzyme that may be conjugated with glutaraldehyde to the bacterial protein streptavidin (AP-SA) and used to detect biotinylated probes that amplify the signal of binding by about a millionfold and thus makes it possible to measure nanogram amounts of proteins using simple UV–VIS detection. Fluorescent, ECL, or other methods have been reported under certain conditions to zeptomole levels of detection [3, 4]. However, most ELISA studies report a limit of detection and or quantification around 1.0 ng/well and so many of the proteins known to be encoded by the human genome, or transcriptome, are far below the detection limit of ELISA.

It has been shown that the combination of the powerful enzymatic amplification by ELISA with sensitive and specific detection of the reaction products by liquid chromatography, electrospray ionization, and mass spectrometry (LC-ESI-MS) results in an ultra-sensitive quantification method termed ELIMSA [5]. The development of ELIMSA might permit the common analysis of attogram amounts of low concentration proteins under highly robust analytical conditions. The enzymatic amplification is obtained from alkaline phosphatase (AP) attached to the specific bacterial probe streptavidin (SA) that binds with high affinity to the chemical tag biotin. The universal chemical tag biotin may be chemically attached to a target molecule by N-hydroxysuccinimide (NHS) ester chemistry or using genetic and biochemical methods. Thus, streptavidin conjugated to the reporter enzyme (AP-SA) may be used to detect NHS-biotinylated antibodies, ligands, or probes with high affinity. There is good reason to suspect that an ELIMSA based on the high affinity of streptavidin–biotin interaction, together with the efficient enzyme alkaline phosphatase and sensitive LC-ESI-MS, should result in ultra-low detection and quantification of ligands, receptors, proteins, or other biological polymers. Enzymatic reactions were previously monitored by mass spectrometry [6], and the alkaline phosphatase substrate pyridoxamine-5-phosphate (PA5P) has been used to generate an ionizable product for ion mobility spectrometry [7]. Moreover, mass spectrometry has been used to confirm the nature of the products from ELISA reactions [8].

Prostate-specific antigen (PSA) is not selective for malignant disease but is a tissue-specific kalikrein protease that indicates benign and cancerous proliferation of prostate tissue and cells [9]. The prostate-specific antigen (PSA) has a concentration distribution from picogram to nanogram amounts in 100 μl of normal human plasma (NHP) from men and so it is often near or below UV–VIS detection in normal patients [10]. The quantification of PSA over the complete range of NHP is challenging by ELISA [10] or mass spectral methods [11], and many normal samples are below the detection limit of the assay. Detection of an increase in PSA over time in normal or prostatectomy patients may be of clinical importance in some cases. In addition, there are excellent experimental reasons to consider PSA and the enzyme alkaline phosphatase as test proteins for developing more sensitive assays: the analyte PSA and the enzyme AP-SA have been used to benchmark the sensitivity of ELISA [12], direct mass spectral detection [11], electrochemical detection [13], and ELIMSA with the substrate naphthol AS-MX phosphate [5] so there are good historical and practical reasons to continue the development of ELIMSA with PSA and AP-SA as the reference analytes. The measurement of PSA is a good model system to show the increased sensitivity provided by ELIMSA to quantify the low abundance samples that might be below 0.1 ng/well [10]. Low abundance ligands, receptors, regulatory factors, and proteins that are specifically expressed in certain tissue types or cells may be useful markers of disease [14].

In this paper, the substrate adenosine monophosphate (AMP) provides important practical advantages over the previous ELIMSA substrate naphthol AS-MX phosphate in that the enzyme product adenosine is basic, ionizes readily but is also hydrophilic, and elutes rapidly with a more symmetrical peak. The isocratic separation of the enzyme product adenosine by normal phase showed a robust and predictable chromatography, is not strongly retained, and shows a flat baseline over the course of measurement. The use of AMP has an important additional advantage in that the enzyme substrate AMP is a common metabolite that is widely available in $^{13}C^{15}N$ isotope labeled form that can be reacted to serve as an internal standard to demonstrate absolute quantification by ELIMSA. To address the issues of accuracy linearity and absolute quantification in ELIMSA by LC-ESI-MS, the experiments were controlled with internal, and external, $^{13}C^{15}N$ labeled adenosine standards to confirm the quantification of the enzyme product. If mass spectrometry is log linear and normal for adenosine, then instead of using UV–VIS spectroscopy to read the 96-well ELISA plate, the production of the colorless nucleoside [15] could be measured using LC-MS with a sensitive ion trap [5]. The substrate AMP is converted by alkaline phosphatase to the basic and hydrophilic molecule adenosine, which resolved well in isocratic LC, ionized very efficiently in an electrospray, and permitted detection to yoctomole amounts of AP-SA on column. The ELIMSA assay for PSA was directly compared to both the gold standard Abbott Architect fluorescence assay and an ECL assay with the same reagents in a blinded experiment that showed good agreement.

Materials and methods

Materials

The model 1100 HPLC was obtained from Agilent Technologies (Santa Clara, CA, USA). The LTQ linear ion trap mass spectrometer was obtained from Thermo Electron Corporation (Thermo Fisher Scientific Inc., Waltham, MA, USA). The 5-μm, 300-Å normal phase silica resin was obtained from Sigma Aldrich (St Louis, MO, USA). The zero volume fittings, frits, and 300 μm I.D. silica and other connectors were obtained from Scientific Products and Equipment (North York, Ontario, Canada). The liquid chromatography was performed with the highest quality solvents that were obtained from Caledon Laboratories (Georgetown, Ontario, Canada). The AMP, adenosine, and adenosine-$^{13}C_{10}$,$^{15}N_5$ 5′-monophosphate sodium salt and all other buffers and salts were obtained from Sigma Aldrich (St Louis, MO, USA) and were of the highest quality available. The NHS–biotin coupling reagent was obtained from Pierce (Thermo Fisher Scientific). The prostate-specific antigen (PSA) capture and detection antibodies were purchased from Medix Biochemica (Kauniainen, Finland). The PSA calibration antigen was obtained from the Scripps Laboratory (San Diego, CA, USA). The alkaline phosphatase–streptavidin (AP-SA) enzyme–probe conjugate was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Immunoassay

The PSA ELIMSA was performed essentially as previously described but with the substitution of the substrate AMP for the substrate naphthol AS-MX phosphate [5]. The AMP substrate, PSA standards, and AP-SA probe were dissolved in water on ice, aliquoted, and freeze dried for day-to-day consistency in the assay. Briefly, the 96-well plates were coated with 250 ng of capture antibody per well in bicarbonate coating buffer prior to blocking the plates in 1 % albumin and 1 % goat serum in 1× PBS pH 7.4 followed by incubating the plasma in 1× PBS, 0.3 M NaCl pH 7.4 for 2 h. All steps including the washes after sample incubation are in PBS with 0.05 % deoxycholate. The sample reaction volume was 100 μl in total volume with 50 μl of plasma+50 μl of 2× buffer (0.6 M NaCl, 2× PBS, 2 % BSA, 2 % goat normal serum) for a final volume of 100 μl of 0.3 M NaCl with 1 % BSA and 1 % goat serum that was incubated for 2 h. The wells were washed 3× with PBS with 0.05 % deoxycholate, 100 μl. Biotinylated detection antibody (2.5 ng/well) was added in 100 μl PBS with 0.05 % deoxycholate with BSA and goat serum. The wells were washed three times with 100 μl of PBS with 0.05 % deoxycholate. A total of 10 ng AP-SA was added in 100 μl PBS with deoxycholate with BSA and goat serum was added for 15 min. The wells were washed rapidly six times with 200 μl of PBS with 0.5 % followed by 3×5 min washes on a tilt table. Then 100 μl of AMP substrate was added in 20 mM Tris and incubated for 90 min prior to dilution of 10 μl with 190 μl of 0.1 % formic acid. A total of 2 μl of the diluted reaction was injected onto normal phase in 70 % acetonitrile 0.1 % acetic acid flowing at 20 μl/min. The PSA ELISA using the high-affinity reagents described here is a robust model system wherein the experimental assay can be first optimized and tested to ensure there is no background binding and confirm the antibodies and calibrants are working and are linear in color assays [5]. The ELIMSA immunoassay was compared to the previously published [16] fluorescent and ECL-based ELISA for PSA [10, 12].

Adenosine standards

The external adenosine standard was created from a powder stock of the adenosine. Additionally, adenosine monophosphate was quantitatively reacted with alkaline phosphatase by adding 1 μg AP-SA per 1 ml standard of 1 mM AMP in 20 mM Tris pH 8.8 enzyme reaction buffer to create an independent external standard. The $^{13}C^{15}N$ adenosine stock was created by adding 1 μg AP-SA per 1 ml standard of 1 mM $^{13}C^{15}N$ AMP in 20 mM Tris pH 8.8 enzyme reaction buffer. The resulting $^{13}C^{15}N$ adenosine internal isotopic standard was added to replicate ELIMSA reactions with $^{12}C^{14}N$ adenosine (i.e., natural adenosine) by isotopic dilution analysis and one-point calibration.

Liquid chromatography, electrospray ionization, and mass spectrometry (LC-ESI-MS)

The standards and samples were diluted 20-fold in 0.1 % formic acid prior to injection of 2 μl via a Rheodyne 7725 manual injector. The analysis was performed with an Agilent 1100 high-pressure liquid chromatography pump in the isocratic mode over a 300 μm I.D.×150 mm normal phase column (5 μm particle diameter, 300 Å pore) in 70 % acetonitrile 0.1 % acetic acid at 20 μl/min via a metal needle electrospray [17] mounted on a linear quadruple ion trap (LTQ XL; Thermo Electron Corporation) [18] as described previously [5]. The trap was set to take the average of four scans each at a fixed length of 200 ms and the automatic modulation was not enabled. The ion intensity for the adenosine ion was monitored at $[M+H]^+$ 268 m/z or for $^{13}C^{15}N$ adenosine $[M+H]^+$ 283 m/z. Extracted results were log transformed and the ion intensity results analyzed for normality and linearity [19–21] using the open source R statistical analysis system.

Blind comparison of the ELIMSA PSA assay to fluorescent and ECL PSA assays showed agreement over a range of plasma concentrations and a linear response from the low

picogram to hundreds of nanograms per well in patients plasma and so covered the wide range of patient samples. The set of samples that represents a wide range of PSA amounts in plasma were assayed by the Abbott Architect commercial PSA ELISA system [16], and the same samples were subsequently analyzed by ELIMSA while blinded and the results were submitted to the analytical center before the results of the fluorescent assay were revealed. Similarly, a set of ultra-low PSA samples from prostatectomy patients were also assayed by ECL [10] and the results kept secret until the results of ELIMSA were submitted to the analytical center. In a separate experiment, a set of female patients were also analyzed by ELIMSA and compared to the previously established range [10].

Results

Adenosine dilution curve

We compared the phosphorylated nucleotides of adenosine, cytidine, uridine, guanosine, and thymidine before and after reaction with alkaline phosphatase. Adenosine $[M+H]^+$ was found to provide an intense signal at 268 *m/z* [15] that was clearly enzyme dependent and so we selected AMP as the substrate for the subsequent studies. The relationship between the adenosine injected and the $[M+H]^+$ ion intensity observed by SIM at 268 *m/z* $[M+H]^+$ was established by a dilution series over a normal phase isocratic LC-MS. The adenosine dilution curve was linear after log transformation and as little as 50 fmol was clearly detectable by LC-ESI-MS (Fig. 1). Adenosine showed a log-linear relationship between quantity versus the intensity observed as measured using LC-MS with single ion monitoring at 268 *m/z* $[M+H]^+$ (Fig. 1a). The plot of log intensity of adenosine $[M+H]^+$ versus log quantity shows that LC-MS of adenosine is log linear to at least low femtomole amounts (Fig. 1b).

Enzymatic reaction

ELIMSA uses the enzyme alkaline phosphatase, so accordingly the signal from the AP-SA probe should depend on enzyme incubation time and enzyme concentration. To confirm if the signal measured in ELIMSA resulted from an enzyme-dependent reaction, we monitored the enzyme product adenosine by SIM 268 *m/z* over time and with respect to enzyme concentration. The time course of adenosine production by AP as measured using LC-ESI-MS with SIM of adenosine $[M+H]^+$ at 268 *m/z* showed that the enzymatic reaction was essentially complete by 3 h (Fig. 2). After log transformation, the intensity of the adenosine peak initially appeared to increase linearly over time, but the rate of adenosine production apparently declined as the reaction approached completion by 3 h of incubation (Fig. 2b). The adenosine ion $[M+H]^+$ intensity monitored at SIM 268 *m/z* was dependent on the quantity of the AP-SA probe (Fig. 3). The enzyme AP conjugated to the bacterial protein streptavidin (AP-SA, average mass ~190,000 g/mol) was diluted down to 1 pg of the conjugate probe per milliliter and then the enzyme was reacted with the substrate AMP to produce adenosine. The striking TIC trace of the enzyme–conjugate curve shows that as little as 1 pg/ml was clearly detected even after injecting only 0.1 μl of the reaction (Fig. 3a). Absolute amounts of the alkaline phosphatase–streptavidin probe (AP-SA) from 1 to 1000 pg/ml were reacted with AMP and the production of the adenosine $[M+H]^+$ ion was measured by injecting 0.1 μl of standards to as low as 1 pg/ml, i.e., 100 attograms of the AP-SA probe, that showed clear signal at SIM 286 *m/z*, that corresponds to 5.26E −22 mol, i.e., 526 yoctomole or about 315 molecules of AP-SA on column. Thus, attogram quantities of AP-SA per milliliter could be detected by monitoring adenosine production from injection that commonly showed R^2 values of ~0.99 and showed a linear response over at least three orders of magnitude after log transformation (Fig. 3b).

ELIMSA PSA assay

After demonstrating that the response to the AP-SA probe was log linear and could be quantified to the equivalent of zeptomoles injected onto the HPLC column by the enzymatic production of adenosine, the method was applied to detect PSA using a biotinylated anti-PSA detection antibody. The PSA assay was linear over three orders of magnitude from 0 to 2.5 ng/well with an R^2 of 0.9918 (p value=2.062E−06) and log transformation resulted in a linear relationship between intensity SIM 268 *m/z* versus picograms of PSA. The assay shows a linear relationship over the picogram range of PSA values with ion intensity values that are well separated from the background and recorded with a high signal-to-noise ratio compared to the small error in the baseline of the TIC trace compared to the height of the TIC trace versus the height of the peaks. The LC-ESI-MS assay was confounded by a slight contamination of the SIM 268 *m/z* channel by some component of the injection buffer resulting in a background peak at 0 pg PSA that must be recorded and subtracted from the SIM 268 *m/z* values that are extracted from the TIC data.

ELIMSA homogeneity and normality

Comparison of populations by ANOVA assumes the random and independent sampling from a normal population. Human plasma PSA values, like many continuous biological variables, may be expected to show a normal distribution within a population. An aliquoted set of NHP

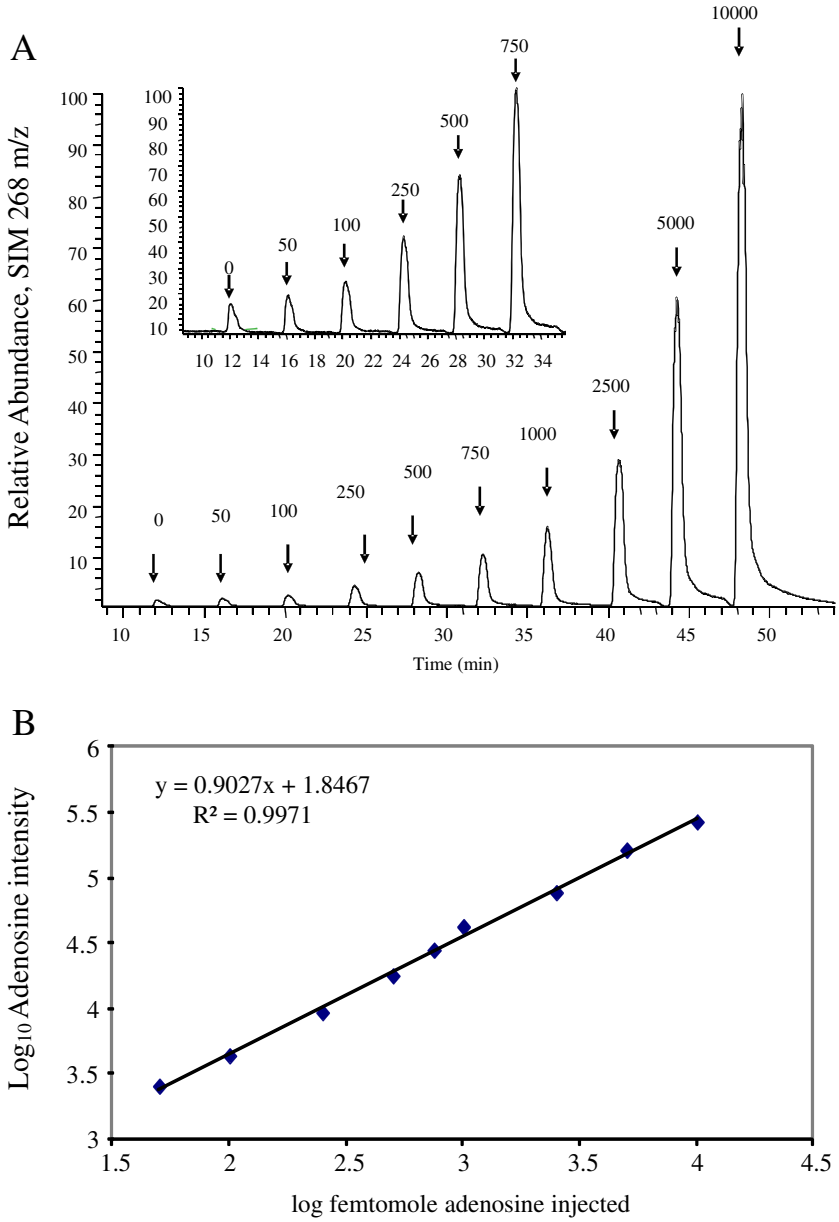

Fig. 1 The relationship between the absolute quantity of adenosine injected and adenosine $[M+H]^+$ ion intensity by SIM at 268 *m/z*. **a** The normal phase isocratic LC-MS chromatogram of the adenosine dilution series where the amount injected on column in femtomole is shown (*inset* shows the detection at low quantity injected); **b** the log-linear relationship between intensity and the log amount of adenosine injected onto the column. Mass spectrometry was observed to be log linear for the analyte adenosine at least over the range shown

samples was used to estimate the distributions of the PSA values obtained from the ELIMSA assay over separate days. The density plot of the PSA values from three independent ELIMSA experiments each on a separate day, and each based on a fresh standard aliquot, showed a Gaussian distribution (see Electronic Supplementary Material). All reagents and samples were aliquoted and the assay showed good reproducibility on three separate days with fresh standards and buffers on each day (Table 1). After calculating the mean and standard errors, it was apparent that the assay showed reasonable variation of ≤10 % within and between days. The quantile plot was used to assess the normality of the entire data set that showed the PSA results from ELIMSA are within the tolerance of the predicted normal distribution (see Electronic Supplement).

Comparison of ELIMSA accuracy and linearity

The PSA standard series apparently resulted in a linear relationship between PSA quantity and intensity of the adenosine $[M+H]^+$ ion as measured by SIM 268 *m/z*. The ELIMSA for PSA depends on the correct estimation of the quantity of adenosine produced in the ELIMSA from the complex of the captured standard or sample PSA with the biotinylated detection antibody that is bound in turn by AP-SA to generate adenosine (Fig. 4), so it is

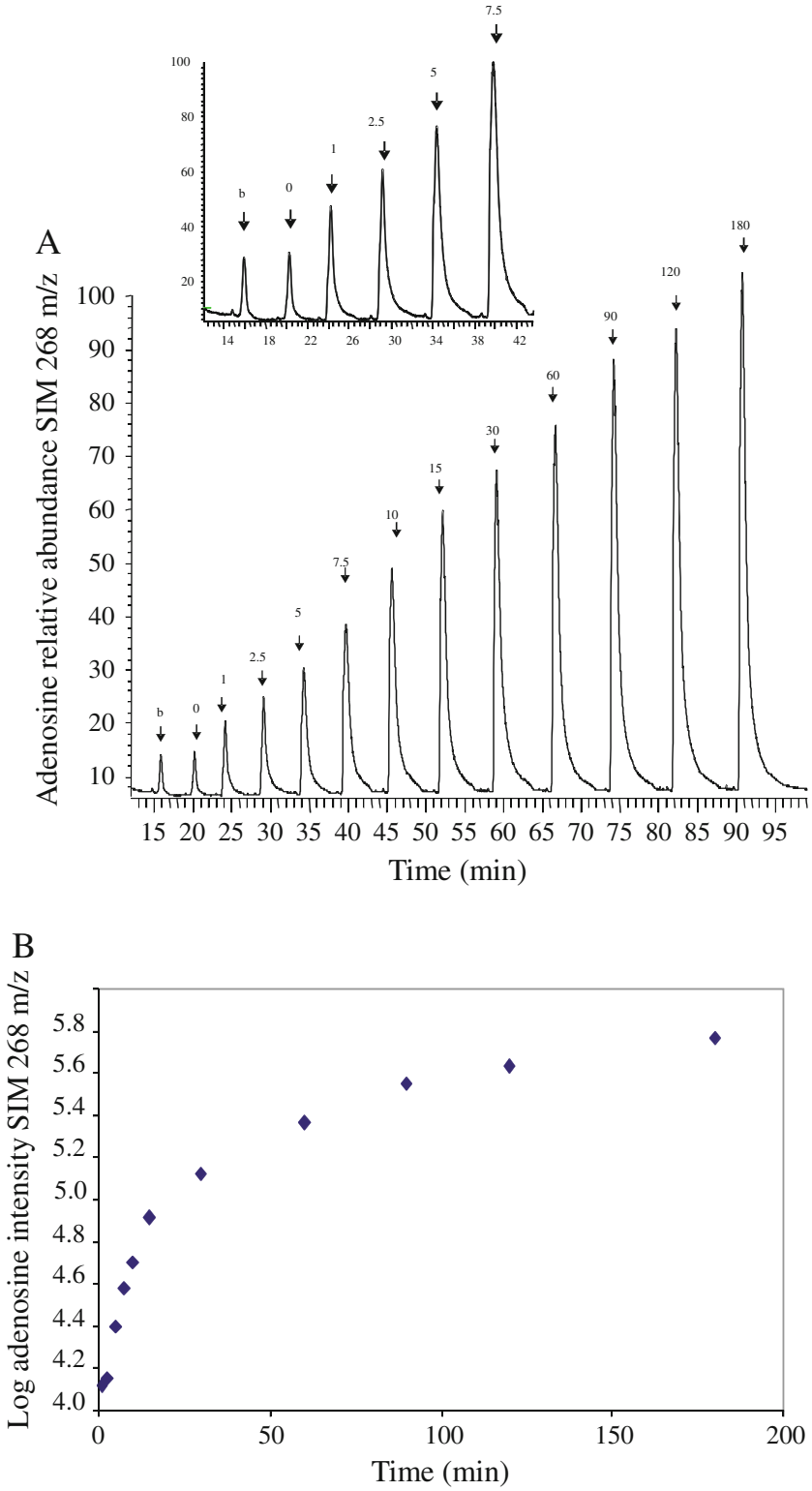

Fig. 2 The time course of adenosine production by AP as measured using LC-MS with SIM of the adenosine ion $[M+H]^+$ at 268 *m/z*. **a** The normal phase isocratic chromatogram showing the time course of the AP enzyme reaction to produce adenosine from AMP with the reaction time in minutes alongside blank (*b*). **b** The plot log intensity SIM 268 *m/z* of the adenosine $[M+H]^+$ ion intensity versus time

important to independently confirm the linearity of the LC-ESI-MS assay for adenosine. The commonly available $^{13}C^{15}N$ AMP was also reacted with excess alkaline phosphatase to create $^{13}C^{15}N$ internal adenosine standards. Thus, the linearity of ELIMSA for PSA is based on the LC-ESI-MS assay for enzymatically released adenosine that was tested and compared to (1) an internal isotope dilution curve, (2) an internal one-point calibration, (3) an

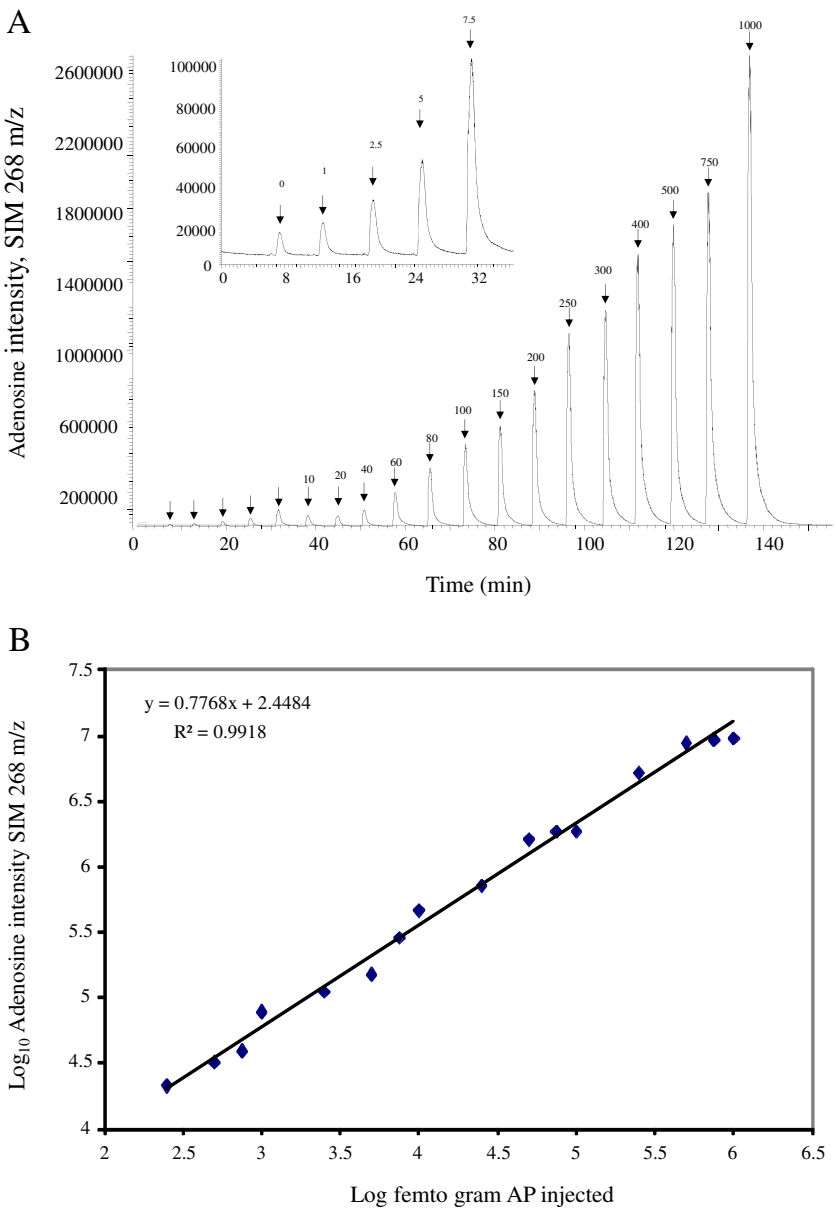

Fig. 3 The linear quantification of picograms per milliliter, or femtogram absolute amounts, of the alkaline phosphatase–streptavidin probe (AP-SA, 190,000 g/mol) by LC-MS with single ion monitoring at 268 *m/z* $[M+H]^+$. **a** The normal phase isocratic chromatogram, showing the concentration of AP from 1 to 1000 pg/ml (*inset* shows detection of as little as 1 pg/ml after diluting the reaction 20-fold in 0.1 % formic acid with injection of 2.0 μl ~5.26E−22 mol on column). **b** The log-linear relationship between AP injected and adenosine intensity over more than three orders of magnitude

external adenosine curve made from the same AMP stock used to standardize the ELIMSA, and (4) an external adenosine curve from dry adenosine powder and the variant of external $^{13}C^{15}N$ adenosine from enzymatic reaction.

Internal isotope dilution curve

The linearity of the PSA assays was measured alongside $^{13}C^{15}N$ adenosine internal isotope dilution curve that showed acceptable linearity after log transformation (R^2>0.99) and indicated that about 0.59 pmol of adenosine was produced by the ELIMSA of the NHP sample (Fig. 4, Table 2).

Internal one-point $^{13}C^{15}N$ adenosine calibration

The isotope dilution curve experiment revealed that the closest standard was 0.50 pmol $^{13}C^{15}N$ adenosine, so this quantity of exogenous standard was added to replicated samples to make the calculation by the one-point calibration method. The average of four NHP adenosine product measurements were used to calculate the picomoles of adenosine at SIM 268 *m/z* from the one-point calibration against an internal standard of 0.50 pmol $^{13}C^{15}N$ adenosine added to the ELIMSA reaction before LC-ESI-MS. The one-point internal calibration method resulted in the estimate of 0.58 pmol adenosine that was produced by the ELIMSA assay (Table 2).

Table 1 The replication of the analysis in normal human plasma to estimate PSA by the AMP substrate ELIMSA is shown in picograms of PSA per well

	Rep 1	Rep 2	Rep 3	
	114.5	155.4	138.6	
	118.1	114.7	133.0	
	140.3	121.9	163.9	
	129.3	145.7	115.4	
	97.2	155.4	177.1	
	178.9	165.7	154.1	
	142.4	176.6	154.1	
	174.0	145.7	192.5	
	175.2	94.1	185.4	
	140.3	92.1	147.8	Grand
Mean	141	136.7	156.1	144.6
Stand Err	8.8	9.3	7.6	5.4

The within-day mean and standard error (Std Err) is shown vertically below and the grand mean and standard error to the right

External adenosine standards

External adenosine standard curves were created from an adenosine stock or from the AMP substrate stock quantitatively reacted with the enzyme AP to yield adenosine and were both used.

The linearity of the external standard of adenosine produced by reacting the AMP with excess AP-SA showed an R^2 of 0.9994 (p value=2.469E−09) and resulted in an estimate of 0.54 pmol adenosine (Table 2). The linearity of the external adenosine standard showed an R^2 of 0.9986 (p value=2.319E−08) and resulted in an estimate of 0.48 pmol adenosine (Table 2).

Blind comparison of ELIMSA to Abbott Architect and ECL PSA assays

The sensitivity of the ELIMSA assay was compared to PSA as measured by the gold standard commercial Abbott Architect fluorescence assay [16] and was also compared to an established ECL assay that showed excellent agreement at PSA levels near or above 100 pg/ml (Fig. 5). However, below about 100 pg/well, the fluorescent method plunged to near zero and no reliable result could be obtained for some samples. In contrast, the ELIMSA method provides values well separated from zero for all samples (Fig. 5). Regression of the ELIMSA results onto the fluorescence results for PSA showed an R^2 of 0.9212 (p value=<2.2E−16). A subsequent comparison of ELIMSA to manual ECL detection [10] also results in good agreement on high abundance samples with an R^2 of 0.9227 for ELIMSA explained by the results obtained from ECL (inset Fig. 5). The ECL system also showed a precipitous drop in signal below about 100 pg, was sometimes not able to quantify all samples, and some patient samples could not be distinguished from zero by ECL, but all samples were clearly separated from the background by ELIMSA. The lowest values from prostatectomy patients and female plasma was about 7 pg/well, and most of these samples were in the range of 10 to 70 pg/well.

Discussion

Advantage of AMP substrate for ELIMSA

It has been suggested that mass spectrometry alone may someday reach the zeptomole range [22], but there have been technical challenges [23]. The use of enzymatic amplification by the conjugated AP-SA probe of the hydrophilic substrate AMP that yields the highly soluble and basic product adenosine was linear, accurate, and reached yoctomole levels of detection. ELIMSA with the AMP substrate was more sensitive and required a lower sample volume compared to electrochemical detection that has previously reached 67 attomol of AP but required at least 50 μl of sample volume [13, 24]. Several technologies have claimed to reach yoctomole levels [25] such as the use of a DNA binding event [26] or the polymerase chain reaction approach [27] where empirical equations extrapolate from the detectable part of the PCR curve to estimate copy number at time zero [28]. However, mass-spectrometry-based assays may utilize high-affinity protein interactions, with isotope labeled internal standards or external standards, to provide absolute quantification and can measure multiple analytes or standards in different m/z channels simultaneously.

Accuracy of ELIMSA for PSA

The ELIMSA approach previously showed close agreement with colorimetric methods across the nanogram per milliliter ranges using the amplex red or naphthol AS-MX substrates [5]. Here, the comparison to the Abbott Architect fluorescence assay showed close agreement of ELIMSA with the previously established assays and demonstrated the ELIMSA was accurate when benchmarked in a blind study that was presumably free from investigator bias. The log-linear intensity results with adenosine here were in agreement with log-linear results from some non-human standard peptides, or tryptic peptides from blood proteins [20], resorufin and naphthol AS-MX phosphate [5], many peptides and proteins from cells [21], or proteins secreted from cells into the experimental media

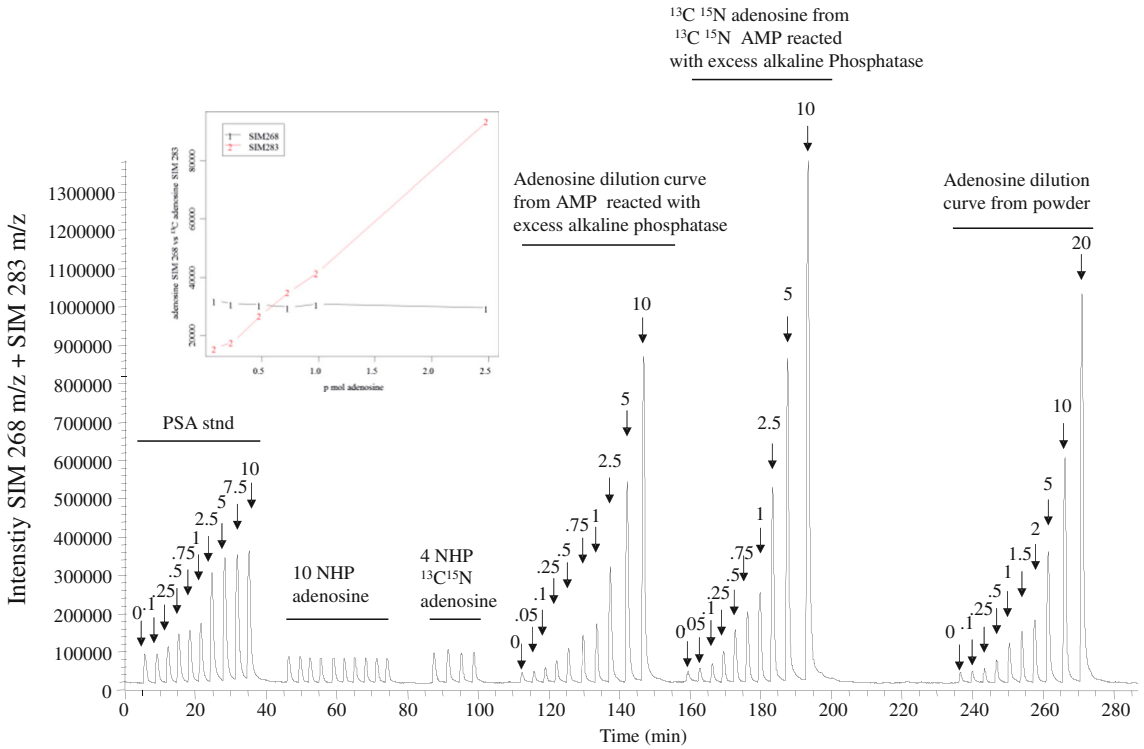

Fig. 4 Isocratic chromatogram showing the total ion current (TIC) trace of ELIMSA alongside a series of external and internal standards. The chromatograph shows experimental results of the comparison of adenosine (SIM 268 *m/z*) produced from the PSA ELIMSA to internal $^{13}C^{15}N$ adenosine (SIM 283 *m/z*) or external adenosine standards diluted from an adenosine stock, or from the reaction of the AMP substrate stock to adenosine by excess alkaline phosphatase–streptavidin probe. The total ion current (TIC) isocratic chromatogram shows the sum of two *m/z* channels added together (SIM 267–269*m/z*+SIM 282–284*m/z*) from which the intensity of the $[M+H]^+$ ions for adenosine at 268*m/z* and $^{13}C^{15}N$ adenosine 283*m/z* are extracted. Chromatogram, *left* to *right*: the ELIMSA PSA standard curve from 0 to 10 ng/well; 10 normal human plasma samples (NHP) measured against the external PSA standards; four NHP samples with internal $^{13}C^{15}N$ adenosine standards (0.5 pmol ^{13}C adenosine SIM 283*m/z*); external adenosine standard from reaction of ELISA AMP substrate stock with excess AP-SA monitored at SIM 268 *m/z*; $^{13}C^{15}N$ adenosine dilution from $^{13}C^{15}N$ ATP completely reacted with AP; external adenosine stock dilution curve from powder stock. (*Inset*—estimation of the production of adenosine SIM 268*m/z* from the ELIMSA after the serial dilution of an internal $^{13}C^{15}N$ adenosine by SIM 283*m/z* across the population of the normal samples producing natural adenosine monitored at SIM 268*m/z*. Reading the average value of SIM 268*m/z* from the replicated reactions off the SIM 283*m/z* standard curve yields an estimate of 0.59 pmol of adenosine produced by the ELIMSA reaction.) The results of the internal and external standard experiments are in Table 2

[19]. The results with the substrate AMP showed trends similar to those of amplex red or naphthol AS-MX phosphate substrates at high PSA levels, and there was good agreement with the fluorescent ELISA at values ≥1 ng/well. The previous results of male normal human plasma by ELIMSA closely match the correct order of magnitude for PSA in control patients reported in the literature and colorimetric assays [5], and the female plasma measured here matched the previously established range [29]. Thus, multiple lines of evidence in agreement indicate that the ELIMSA standardized against absolute amounts of the PSA calibrant provides the accurate absolute quantification of PSA. ELIMSA may be used to accurately quantify proteins to at least picogram levels of PSA and will probably reach greater levels of sensitivity that will be mostly limited by factors specific to each experimental set up including detergents, blocking agents, and LC-ESI-MS apparatus.

Accuracy and linearity of LC-ESI-MS for adenosine and $^{13}C^{15}N$ adenosine

The results with the internal $^{13}C^{15}N$ isotope dilution curve were in close agreement with the external adenosine standard indicating the LC-ESI-MS assay for adenosine was linear and accurate. The one-point calibration method, which assumes linearity, was also in close agreement with the internal isotope dilution curve and the adenosine external standard. Hence, four independent methods were within the same order of magnitude and within the same digit so in general showed excellent agreement that ELIMSA increases the sensitivity of LC-ESI-MS/MS for PSA by about a millionfold. The unique capacity of mass spectrometry to demonstrate the accuracy of the absolute quantification by internal and external standards clearly shows that the LC-ESI-MS approach is the most sensitive, flexible, linear, and accurate method of quantification with major advantages over technologies that cannot

Table 2 A comparison of the estimated production of adenosine by ELIMSA to (1) an internal isotope dilution curve, (2) an internal one-point calibration, (3) an external adenosine curve made from the same AMP stock used to standardize the ELIMSA, and (4) an external adenosine curve from dry adenosine powder

Method	Result
$^{13}C^{15}N$ adenosine internal isotope dilution	0.59 pmol A
External curve from reacted AMP stock	0.54 pmol A
Adenosine powder external curve	0.48 pmol A
$^{13}C^{15}N$ PA internal ratio	0.58 pmol A
PSA external curve	0.116 ng PSA

The internal isotope dilution was made with $^{13}C^{15}N$ AMP that was enzymatically converted to $^{13}C^{15}N$ adenosine. Similarly, an external standard curve of adenosine was prepared from the AMP substrate stock after complete reaction with 1 μg AP-SA per 1 ml standard of 1 mM AMP. The internal one-point calibration was made by the exogenous addition of 0.50 pmol of $^{13}C^{15}N$ adenosine to the ELIMSA. The four independent methods for estimating adenosine production from the ELIMSA of the same NHP sample measurement shown were within one digit in the same order of magnitude, indicating that the externally calibrated LC-ESI-MS assay of 0.116 ng PSA per well based on the release of adenosine is reasonably linear and accurate

discern stable isotopes or technologies that require radioisotopes. The isotope dilution curve here and the internal isotope one-point calibration experiments showed close agreement with external standard curves after log transformation [21] and thus unambiguously demonstrated that mass spectrometry does not necessarily show a complex relationship between intensity and quantity [19, 20] as previously assumed [30].

Sensitivity of ELIMSA

The ELIMSA was able to measure the PSA levels in all normal, female, and prostatectomy samples and showed greater sensitivity than the fluorescent or ECL approaches. The direct quantification of the generic AP-SA molecular probe by LC-ESI-MS permits the routine analysis of receptor–ligand complexes with quantification of biological molecules to femtogram, i.e., zeptomole, amounts. The PSA ELIMSA reached to the order of 1 pg/well and could detect and quantify PSA that was well below the range of colorimetric, fluorescent, or ECL-based assays. The ELIMSA method was required to make reliable measurements below 100 pg/well, and both fluorescence and ECL failed to detect many of the plasma PSA levels in the low picogram range. The demonstrated measurement of AP-SA to the yoctomole range is as low a quantity as the most sensitive methods claimed to date [4, 11–13, 24, 31–40], but uses high-affinity protein interactions that may be directed against small but unique epitopes and commonly available ELISA and micro-electrospray that is robust, reproducible, and portable across mass spectrometric platforms. Even in the most challenging samples such as prostatectomy and female plasma, all PSA values were in the

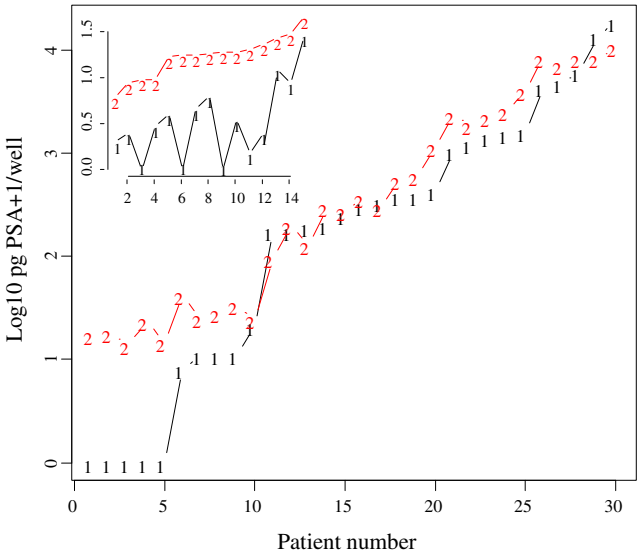

Fig. 5 Blind comparison of the ELIMSA detection method for the PSA assay in human plasma samples to ELISA for the quantification of PSA by fluorescence and ECL. *Main panel*: a wide range of PSA values to the hundreds of nanogram range from automated Abbott Architect fluorescence assay for total PSA (*1*) versus the ELIMSA detection (*2*) of the same samples over 30 patient samples ranked by the fluorescence results; the ELISA results dropped precipitously below 100 pg/well and was not able to differentiate many samples from zero; in contrast, ELIMSA could quantify all samples. [*Inset*—ECL assay (*1*) versus ELIMSA (*2*) of prostatectomy patient plasma where the arbitrary patient number was sorted by the results of ELIMSA; all of the prostatectomy samples, including those that could not be separated from the background by fluorescence or ECL, were robustly quantified by ELIMSA]

zeptomole range on column and could be robustly quantified. After purposefully selecting plasma samples that contain very low or undetectable amounts of PSA by colorimetric or ECL assays, it was possible to show detection of PSA to as low as on the order of ~1 pg/well or less. Hence, the ELIMSA method showed that in practical terms all patient PSA levels are now within the limit of quantification. The use of LC-MS to measure enzyme-conjugated molecular probes will permit the quantification of biological molecules at picogram amounts or less with high signal-to-noise values and essentially means that the sensitivity of the detection method is no longer the limiting factor in biological mass spectrometry but rather only the availability of high-affinity reagents. The results indicated that LC-ESI-MS with the AMP substrate may detect yoctomole amounts of the universal AP-SA probe–enzyme conjugate and quantify zeptomole amounts of a protein.

Utility of ELIMSA

PSA exists at low levels in human blood, and many samples have levels that may be below detection by ELISA or LC-ESI-MS/MS. Many transcription factors, cellular receptors or agonists, and cell signaling proteins including oncogenes and

receptors that may play a regulatory role in cell and tissue differentiation [14] have been reported in blood after partition chromatography of the intact proteins prior to tryptic digestion chromatography or by selective extraction of endogenous peptides prior to LC-ESI-MS/MS [41–43]. Many of the factors identified from blood near the edge of detection and identification by LC-ESI-MS/MS remain below the limit of confident quantification by present mass spectral technologies [44]. Ultra high-affinity ligands such as cytokines, chemokines, interferons, interleukin, tumor necrosis factors, and many other potent regulatory proteins that effectively bind their target receptors even at femtomolar or attomolar concentrations remain far below any credible quantification by mass spectrometry alone. However, the combination of enzyme amplification of the signal from the biotinylated probe together with mass spectrometry shows excellent prospect to achieve a Gaussian linear quantification with good signal-to-noise ratios over the entire range of physiologically relevant concentrations [5]. Hence, where high-affinity reagents are available, the use of ELIMSA will ensure that the sensitivity of the detection methods will no longer be the limiting factor in the non-radioactive analysis of biological molecules.

Contributions

AF-M performed ELIMSA experiments and numerical summaries and proofed the paper; EPD designed the comparison of ELIMSA versus the fluorescent assay for the normal patients and the comparison of ELIMSA versus ECL for the prostatectomy patients that were performed by AS; JD performed the SDS–PAGE; JGM conceived and designed the ELIMSA experiment, performed some experiments, created the charts and graphs in R, and wrote the paper.

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