Employment of 4-(1-imidazolyl)phenol as a luminol signal enhancer in a competitive-type chemiluminescence immunoassay and its comparison with the conventional antigen–horseradish peroxidase conjugate-based assay

Yannis Dotsikas, Yannis L. Loukas

Department of Pharmaceutical Chemistry, School of Pharmacy, University of Athens, Panepistimioupoli, Zografou, GR-15771 Athens, Greece

Received 4 July 2003; received in revised form 14 November 2003; accepted 4 December 2003

Abstract

This study describes the employment of a novel imidazole-substituted phenol (4-(1-imidazolyl)phenol) as a highly potent signal enhancer in a horseradish peroxidase (HRP)–luminol chemiluminescence (CL) immunoassay. This competitive-type immunoassay for the model antigen fentanyl is based on the use of fentanyl polyclonal antibody immobilized on white microtiter plates and a biotinylated bovine serum albumin (BSA)–fentanyl derivative as a tracer. The latter was detected by means of streptavidin labeled with HRP, resulting in the generation of a high-intensity and relatively stable chemiluminescent signal, immediately after the addition of the substrate solution (NOAS). The developed method fulfilled the requirements of accuracy (percentage recovery ranged from 93.8 to 107%) and precision (intra- and inter-assay CVs were 2.5–5.2 and 4.5–11.9%, respectively). Its plasma detection limit (1.05 pg ml$^{-1}$) was lower than those of previous immunoassays. The novel assay was compared in terms of sensitivity and concentration range with other common HRP substrate systems: luminol–$p$-iodophenol–$H_2O_2$ and TMB–$H_2O_2$. Finally, the described method was compared with an HRP–fentanyl conjugate-based assay, similar to commercially available kits (SKIT), employing the novel substrate solution for both assays and the differences observed were explained by applying previously described models. The detection limit was 4.82 pg ml$^{-1}$ for SKIT, recovery values were 94.2–105% and intra- and inter-assay CVs were 2.5–5.2 and 4.5–11.9%, respectively. In conclusion, the proposed assay could be utilized for a wide range of molecules and replace the existing enzyme-labeled antigen-based kits.

© 2003 Elsevier B.V. All rights reserved.

Keywords: 4-(1-Imidazolyl)phenol; Luminol enhancer; Chemiluminescence; Immunoassay; Biotinylated conjugate; Streptavidin; Horseradish peroxidase

1. Introduction

Chemiluminescence enzyme immunoassays (CLEIAs) have received great attention in recent years. Horseradish peroxidase (HRP) is the enzyme that has gained the greatest significance in analytical biochemistry for the determination of hydrogen peroxide and other compounds through coupled enzymatic reactions [1,2] leading to the development of very sensitive CLEIAs. HRP catalyzes the reaction between a hydrogen acceptor (oxidant), such as hydrogen peroxide, and a hydrogen donor including chemiluminescent substrates such as luminol. The latter compound when oxidized emits light energy because of the decay of the excited-state oxidation product. The intensity of the emitted light can be significantly increased by addition of an enhancer such as $p$-siodophenol (PIP) [3–5], which has emerged as the most popular enhancer of the HRP-catalyzed chemiluminescent oxidation of luminol. Enhanced chemiluminescent reactions provide more intense, prolonged and stable light emission [6]. A variety of substituted phenols, e.g. firefly luciferin, 6-hydroxybenzothiazole derivatives [7,8], and substituted arylboronic acid derivatives, e.g. 4-iodophenylboronic acid [9] or more complicated ones [10], have been applied as luminol signal enhancers.

In the present study a novel enhancer was employed with an imidazole molecule as substituent at the 4-position of the phenol (Fig. 1): 4-(1-imidazolyl)phenol (4-IMP). This difference in the 4-substituent seems to play a critical role in
the enhancement efficiency of phenol derivatives. Initially, a series of experiments were performed to find optimum conditions of CL in the presence of 4-IMP. Next, comparative studies between PIP and 4-IMP were undertaken which indicated the differences in CL intensity and light kinetics when each enhancer was utilized. These experiments demonstrated that 4-IMP provided a more potent signal than PIP did, but also a more rapid signal decay.

This substrate system of HRP was employed in an immunoassay that has been previously described [5] where TMB, a colorimetric substrate, and luminol with PIP had been used. The novel assay for the model antigen "fentanyl", a potent narcotic analgesic, that we developed, enabled us to measure even less-concentrated plasma samples, than the previous assays allowed. The use of 4-IMP instead of PIP led to a decrease of the detection limit of the assay by a factor of 4.5, which was even lower than the respective from fentanyl radioimmunoassays (RIAs) [11–13].

The presented assay has the major advantage of its simplicity. The strong interaction between biotin and streptavidin was utilized to gain binding between a biotinylated fentanyl–BSA derivative and a streptavidin–HRP conjugate. The addition of the substrate solution results in the generation of a very intense signal for each tracer molecule, which is directly proportional to the amount of the associated marker enzymes. This number is the critical factor that leads to decreased detection limits regarding with the classical ELISA kits, where there is only one enzyme per tracer (fentanyl–HRP). We compared both assays in terms of sensitivity and concentration range, by applying the novel substrate solution and we used previously described models in an attempt to explain the differences and especially the superiority of the presented assay.

2. Experimental

2.1. Reagents

Anti-fentanyl rabbit polyclonal antibody (9.2 µmol/l) and fentanyl–BSA conjugate were obtained from Biostride (USA). Fentanyl–HRP derivative (12 µmol/l) was purchased from USBio (Europa Bioproducts, UK). Sulfo-NHS-LC-Biotin was obtained from Molecular Probes (Eugene, OR) and BSA (RIA and ELISA grade) was brought from Calbiochem (Germany). TMB peroxide substrate solution was obtained from Pierce (Rockford, IL, USA). Hydrogen peroxide (3%, w/v), p-iodophenol 99% and 4-(1-imidazolyl)phenol 97% were obtained from Aldrich (Germany). Streptavidin labeled with type VI peroxidase, luminol, tris(hydroxymethyl)aminomethane (Tris) and all other reagents were from Sigma (Germany). All aqueous solutions and buffers were prepared using water de-ionized and doubly distilled (resistivity > 18 MΩ cm).

The washing solution used in this protocol was a PBS buffer (pH 7.4) containing 0.05% (v/v) Tween 20 and the assay buffer was the washing solution containing 0.1% (w/v) BSA and 2% sodium salicylate as displacing agent [14,15] of fentanyl from plasma proteins [16]. Dilutions of conjugates and standards were made using the assay buffer. The coating buffer consisted of a 0.1 M carbonate-bicarbonate buffer (pH 9.6) and the blocking solution was a PBS buffer containing 1% BSA (w/v). Fentanyl solutions were kept in polystyrene tubes, due to its adsorption onto glass surface.

2.2. Instrumentation

All measurements were performed with a Fluostar Galaxy (BMG Lab Technologies, Germany) multifunctional microplate reader. Luminescence optics was installed for the experiments and all emitting light was recorded without any emission filter. The 96-well microtiter plates (opaque white polystyrene plates with a Maxisorp surface, which exhibited low cross-talk between adjacent wells) were obtained from Nunc (Nalge Nunc, UK). All plates were washed with a fully automated Tecan Columbus (Tecan, Austria) 96-well microplate washer.

2.3. Immunoassay procedures

Both concepts of the novel assay (NOAS) and the one similar to kit (SKIT) are schematically presented in Fig. 2. For each one, the wells of the microtiter plates were filled with 100 µl of fentanyl polyclonal antibody dilution (1:2000 for NOAS and 1:22000 for SKIT) in coating buffer and incubated for 14 h at 4 °C. Next, the antibody solution was removed and the plates were post-coated with 200 µl of blocking solution for 1 h at room temperature.

Fig. 2. Schematic illustration of the concept for the proposed chemiluminescence-based immunoassays.
After being washed four times with 300 μl of washing solution, the microwells were filled with 50 μl of fentanyl plasma standard solutions (0–2400 pg ml⁻¹ for NOAS and 0–3200 pg ml⁻¹ for SKIT) or plasma samples and 50 μl of the diluted (1:900) biotinylated fentanyl–BSA conjugate in assay buffer for NOAS and incubated for 1 h at room temperature. In the case of SKIT, the previous conjugate was replaced by a diluted solution (1:1100) of fentanyl–HRP conjugate in assay buffer. The wells were rewashed six times by means of the same washing solution and 100 μl of streptavidin–HRP dilution (1:7500 from a stock solution 125 mg l⁻¹) in assay buffer was dispensed and incubated for 30 min at room temperature for NOAS. After washing once again six times, CL development was carried out by adding 150 μl of luminol substrate solution in both assays and the emitted photons were measured immediately, since a decrease in signal intensity [17] was observed.

The biotinylated fentanyl–BSA conjugate was prepared as previously described [5]. In brief, Sulfo-NHS-LC-Biotin, a water-soluble biotinylation reagent, was added to an aqueous solution (0.1 M Na₃PO₄, 0.15 M NaCl, pH 7.2) of fentanyl–BSA conjugate in a molar ratio 20:1 (biotin:conjugate). After an incubation of 2 h, the biotinylated derivative was purified by gel filtration on Sephadex G-25 (mobile phase: PBS, pH 7.4) to remove unreacted fentanyl–BSA conjugate, excess of biotinylation reagent and other low-molecular-mass by-products. Then, the biotinylated derivative was detected by spectrophotometric measurement at 280 nm. The concentration of the biotinylated tracer was 4.8 μmol l⁻¹.

2.4. Chemiluminescence substrate

A freshly prepared luminol substrate solution was added to each well of both assays with a multichannel pipette, consisted of luminol (0.1 mmol l⁻¹), hydrogen peroxide (1 mmol l⁻¹) and 4-(1-imidazolyl)phenol in Tris buffer (0.1 M, pH 8.5). The typical profile for a CL transient signal depends on the generation rate of the light-emitting product and the formation rate of the final product [18]. The CL emission was expected to reach a maximum quickly after initiation, remain stable for a while and then decay slowly under these analytical conditions. Measurements were carried out by using a continuous measurement cycle and substrate solution addition was performed manually (off board).

2.5. Antibody titration

The titration curve is used to determine the saturating and separating titters of the antibody (Ab) for a specific tracer. Saturating titter is the point of the titration curve at which the signal intensity (e.g. absorbance, CL intensity, etc.) does not change significantly (plateau) with increasing titter (antibody dilution). Separating titter is defined as the dilution of antibody that gives a signal intensity close to half of the maximum obtained (~50%, midpoint of the titration curve). This value falls on the linear part of the curve (sigmoid) where significant changes in signal intensity are observed with increasing titter. This value is used as the optimal antibody dilution for the development of a novel immunoassay and for the assessment of the affinity between antibody and tracer [19].

In the present study, two titration curves were obtained corresponding to the two available fentanyl conjugates. The following antibody dilutions were used in triplicate to cover the surface of the micotiter plates: 1:500, 1:1000, 1:5000, 1:10 000, 1:50 000, 1:100 000 and 1:1 000 000. Next, the wells were post-coated with blocking solution, as mentioned above. Both fentanyl conjugates were then added in the same volume with the antibody solution. In the case of fentanyl–BSA derivative, the same volume of a 5000-fold diluted streptavidin–HRP was added to each well. It should be noted that washing steps took place after every addition and incubation. Finally, the wells were filled with the novel substrate solution and CL signal was measured immediately.

3. Results

3.1. Chemiluminescence intensity and kinetics

The effect of 4-IMP concentration on CL intensity, measured in relative luminescence units (RLU), is shown in Fig. 3. The major signal was observed applying the novel enhancer at 0.2 mmol l⁻¹. Based on previous studies [20,21] with different 4-substituent phenols, we surmised that the imidazole analogue would be a more potent enhancer than PIP in a chemiluminescent assay for this enzyme. To this purpose, six wells were filled with 20 μl of HRP solution (0.2 mmol l⁻¹), followed by the addition of the two substrate solutions in triplicate: 4-IMP and PIP [5] solutions in optimum conditions. The obtained maximum signal by 4-IMP

![Fig. 3. Effect of 4-IMP concentration on the CL intensity.](image-url)
had a 2.5-fold increase against the respective signal from PIP.

As for the kinetics of light emission, Figs. 4 and 5 show CL emission for PIP and 4-IMP reaction, respectively. Maximum signal was reached within 60 s after initiation, stayed essentially stable for the next few minutes (4 min for 4-IMP and 8 min for PIP) and then decayed slowly in both cases. However, it is obvious that CL intensity had a more rapid decay in the case of 4-IMP than in the case of PIP. CL measurement does not require in situ initiator addition owing to the relatively long-lived nature of the signal.

3.2. Detection limits

The obtained signals of the fentanyl plasma standard solutions, using 4-IMP as signal enhancer, were fitted by means of the four-parameter logistic equation [22] for both assays (Fig. 6). The standard curves were plotted as mean $\frac{B}{B_0}$ against the logarithm of the antigen concentration, where $\frac{B}{B_0}$ was the signal obtained divided by the mean signal obtained for the zero standards and ranges from 0.0 to 1.0. The minimum detectable concentration was defined as the concentration of fentanyl corresponding to the signal of the zero plasma standard minus 3 standard deviations of the same zero standard. The detection limits were calculated to be 1.05 pg ml$^{-1}$ for NOAS and 5.12 pg ml$^{-1}$ for SKIT.

3.3. Precision and accuracy

For NOAS the intra- and inter-assay CVs ranged from 2.5 to 5.2 and 4.5 to 11.9%, respectively, while for SKIT intra- and inter-assay CVs were 1.8–4.3 and 4.2–9.3%, respectively, demonstrating an acceptable level of precision for both assays. The ranges of fentanyl concentrations used for the evaluation of precision and accuracy were 0.800–2400 pg ml$^{-1}$ for NOAS and 4.00–3200 pg ml$^{-1}$ for SKIT.

Accuracy was evaluated by adding increasing amounts of fentanyl to plasma samples and measuring the percentage recovery. The mean percentage recovery values ranged from 93.8 to 107% for NOAS and 94.2 to 105% for SKIT, and were not significantly affected by the dose.

3.4. Estimation of antibody–conjugates affinity

In Fig. 7 the titration curves for biotinylated fentanyl–BSA and fentanyl–HRP conjugates for fentanyl Ab are presented. The changes of signal intensity during Ab titration were fit...
to a four-parameter equation, given by \[ \frac{B}{B_0} = \beta_1 + \frac{\beta_2}{1 + (\log Ab/\beta_3)^{\beta_4}} \]

where \( B/B_0 \) is the signal obtained for each Ab dilution \( (\log Ab) \) divided by the maximum signal obtained for the most concentrated Ab dilution. \( \beta_1 \) and \( \beta_2 \) are the asymptotic values as \( \log Ab \to 0 \) and \( \log Ab \to \infty \), respectively. \( \beta_3 \) is the predicted \( \log Ab \) value at the midpoint of the titration curve (half of maximum value) and \( \beta_4 \) is related to the slope. The \( R^2 \) values for the fit were 0.996 and 0.993 for the biotinylated fentanyl–BSA and fentanyl–HRP conjugates, respectively, indicating a very good correlation between equation and experimental values.

The parameter \( \beta_3 \) can be used as a measure of the affinity for each fentanyl conjugate binding to the fentanyl antibody. Using this method, the apparent dissociation constants, as indicated in Fig. 7 by \( X_1 \) and \( X_2 \), were determined to be 3.26 and 0.58 nm for biotinylated fentanyl–BSA and fentanyl–HRP conjugates, respectively.

4. Discussion

Chemiluminescence detection based on the oxidation of luminol by hydrogen peroxide catalyzed by HRP, provides a convenient as well as sensitive system for application in immunoassays. The presence of a phenol derivative results in a significant increase in the intensity without losing the relatively long-lived nature of the signal, due to the capacity of HRP to repeatedly cycle the reaction from substrate to product. As reported in the present study, the addition of a new enhancer, 4-(1-imidazolyl)phenol, produced 2.5 times greater degree of enhancement than the classical enhancer p-iodophenol. A comparison of calibration curves for the same immunoassay system (NOAS) for the two enhancers [5] is demonstrated in Fig. 8. It is obvious that just the addition of the novel enhancer led to a decrease in the detection limit from 4.80 to 1.05 pg ml\(^{-1}\).

The precise mechanism of the HRP-catalyzed chemiluminescent oxidation of luminol in the presence of a phenol derivative remains unknown [21]. However, it has been proposed that a pathway involving HRP intermediates and the generation of the phenoxy radical of the enhancer, followed by the reaction of the phenoxy radical with luminol is a major element in the whole process [24]. It is also assumed that enhancer radicals can be expected to affect the CL intensity via (i) the rate of the enzyme turnover and (ii) the electron transfer between enhancer radicals and luminol [25]. One factor that is critical is the stability of the enhancer radicals which is mainly dependent on the substituents at the 4-position of the phenols [24,26,27]. The electronic properties of the enhancer have a great influence on the reduction potentials of the enhancer radicals, and hence CL intensity can vary a lot for a number of phenol derivatives. The presence of an imidazolyl group at the 4-position of the phenol has a more stabilizing effect on the stability of the enhancer radical than an iodo group and therefore the superiority of 4-IMP against PIP on the CL enhancement effect can be easily explained.

4-IMP had two effects: it increased the light emission from the luminol–H\(_2\)O\(_2\)–HRP reaction more effectively than PIP did and decreased the time period that the light emission can be considered as stable. In other words it increased light decay. As it was reported earlier [28], the enzyme inactivation is the main reason for light decay in the course of the reaction. The loss of enzyme activity can be partially explained by nonspecific interaction of radical species (including phenoxy radicals) with protein globule. 4-IMP radicals are more stable than PIP radicals and thus can cause
a higher increase in CL intensity and signal decay. Experiments were performed under the same HRP levels, since at very high HRP concentrations the intensity versus time profile changes dramatically and the decay is much faster [29]. Also, it should be stated that all HRP isoenzymes do not have the same ability in enhancing CL intensity. It has been proven that type VEA, which was used in the current experiments, demonstrated the greatest effect [10].

The employment of the novel enhancer resulted in a significant decrease in the detection limit of a previously described immunoassay for fentanyl [5]. Specifically, the detection limit in NOAS was estimated to be 1.05 pg ml\(^{-1}\), while the replacement of 4-IMP with PIP gave a detection limit of 4.82 pg ml\(^{-1}\). CL immunoassays have inherently lower detection limits than the respective based on UV absorption measurement, hence the relatively high value of the detection limit of the assay having TMB as the colorimetric substrate of HRP compared to NOAS (45.1 pg ml\(^{-1}\)) seems logical [5].

The employment of antigen–BSA derivatives instead of the conventional antigen–HRP derivatives in the recent years [30], seems to have certain advantages. In the present study, the fentanyl–BSA was biotinylated according to a protocol previously described [31]. The biotinylated tracer possessed multiple haptens (~20) and additionally 12, on average, bioin molecules were covalently attached per conjugate, as it was previously estimated [5]. The hapten is typically linked to the carrier protein through a lysine residue. Since the original immunogen used for antibody production had great conformational similarity with the hapten conjugate, it is not unusual that such a bulky antigen derivative can effectively compete with the unlabeled hapten conjugate, by virtue of having some HRP molecules coupled to one molecule of the derivative, via streptavidin bridge, in contrast to one HRP molecule in the conventional fentanyl–HRP derivative, performed better on two counts: (i) it diminished the detection limit by a factor of 4.9 (from 5.12 to 1.05 pg ml\(^{-1}\)) and (ii) provided a more rapid slope of the calibration curve. One can argue that a greater improvement should occur. However, the difference in size had an effect on the conjugate–Ab affinity (different secondary forces and lateral interactions [33]) and the binding of one conjugate to the Ab on the plate surface renders some neighboring Ab molecules spatially unavailable for binding, as it was successfully described in a similar occasion with mathematical models [34].

5. Conclusions

The previously reported approach for the development of a novel competitive immunoassay by using an antigen–BSA conjugate as a tracer led to a significant improvement in detection limit in comparison with the corresponding conventional ELISA for the model antigen “fentanyl”. It is well known that the tracer plays a critical role in the functionality of a competitive immunoassay and it seems that BSA can replace HRP or other enzymes as a carrier protein with the requirement to attach a label, e.g. by biotinylation and adding streptavidin labeled with HRP in an additional step. Since the number of the associated enzymes per conjugate is much higher than for classical antigen–HRP derivatives, the problem of the bulky size of the novel tracer that affects its approach to the immobilized antibody can be overcome.

Moreover, the employment of a novel phenol derivative, 4-(1-imidazolyl)phenol, resulted in a further improvement of the detection limit, allowing the measurement of very low-concentrated plasma samples. The magnitude of the signal enhancement was about 2.5-fold in comparison with the classical enhancer (p-iodophenol) in a luminol–H\(_2\)O\(_2\)–HRP system. However, the novel substrate solution demonstrated a less stable signal after the initiation of the reaction, hence the measurement should be performed immediately.

References


学霸图书馆
www.xuebalib.com

本文献由“学霸图书馆-文献云下载”收集自网络，仅供学习交流使用。

学霸图书馆（www.xuebalib.com）是一个“整合众多图书馆数据库资源，
提供一站式文献检索和下载服务”的24小时在线不限IP图书馆。
图书馆致力于便利、促进学习与科研，提供最强文献下载服务。

图书馆导航：
图书馆首页 文献云下载 图书馆入口 外文数据库大全 疑难文献辅助工具