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Direct monitoring changes of salbutamol concentration in serum by chemiluminescent imaging

Canli Zhang [a], Ruichao Zhang [a], Na Na [a,*], Joris R. Delanghe [b], Jin Ouyang [a,*]

[a] College of Chemistry, Beijing Normal University, Beijing 100875, PR China
[b] Department of Clinical Chemistry, Microbiology and Immunology, University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium

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ABSTRACT

We report in this manuscript, the use of direct ammonium persulfate-enhanced chemiluminescence (CL) imaging, to monitor changes to measure serum salbutamol concentration in subjects of different haptoglobin (Hp) phenotypes at different dosing time. It was noted that CL generated from Hp was decreased due to salbutamol's reducibility, which was used for monitoring salbutamol concentration in serum. The serum from the subjects treated by oral administration of salbutamol, was collected at different dosing time and was separated by polyacrylamide gel electrophoresis (PAGE) prior to the CL detection. According to CL images, samples were separated into three groups based on the Hp phenotypes. The curves of CL signal intensity versus time were obtained for each group, and we demonstrated that there were more significant variables on binding ability between groups. The maximum salbutamol concentration in the serum appeared after 4 h, which was in agreement with the literature. In addition, the binding constants of salbutamol to Hp were determined by a fluorescence-based method, whose results were in agreement with the phenomenon of the greater salbutamol metabolism rate for Group Hp 1-1 than Group Hp 2-2. The presented method can monitor changes of salbutamol concentration in serum directly, making the procedures much simple, convenient, rapid and has the property of lower cost. It provided us with excellent reference information for the individual dosage regimen of different Hp groups, which hopefully could become a potential method for further pharmaceutical research.

1. Introduction

Salbutamol, also known as albuterol, is a widely used β_2-adrenoceptor agonist for treatment of bronchial asthma, chronic obstructive lung disease, and other allergic diseases associated with respiratory pathway of humans or animals [1]. The use of salbutamol is forbidden by the oral route due to an anabolic-like effect as well as a strong adrenergic stimulation [2]. However, individual patients need different quantity of salbutamol for producing a therapeutic response because there is individual variability in pharmacokinetic response for a known plasma concentration [1,3]. Therefore, monitoring the changes of salbutamol concentration in serum is quite important not only in the sports testing but also in individual difference research for dose administration.

A variety of analytical techniques can be used for monitoring changes of salbutamol concentration in serum, such as high performance liquid chromatography [4], gas chromatography [5], ion chromatography [6], capillary electrophoresis [7,8], fluorescence spectrometry-related method [9], square wave voltammetry [10], and mass spectrometry [11–13]. However, these techniques are time consuming, tedious and require complicated pre-treatment procedures, and the high cost due to the adopted expensive instrument. Thus, the development of a simple method for rapidly monitoring the changes of salbutamol concentration versus dosing time is still desirable.

Recently, the direct CL detection of biomolecules in gels has been reported. This method can be used to analyse samples directly in complicated matrix, and it avoids the separation of the drug from the matrix which makes the detection much simpler and rapider [14]. We recently reported the rapid enhanced CL detection method for the Hp phenotypes, following PAGE. This method used ammonium persulfate to enhance the CL signal generated from the reaction between luminol and hydrogen peroxide [15]. Because this kind of CL generation reaction was an oxidation reaction, the reductants would decrease the CL signal intensity. Due to the presence of phenolic hydroxyl group in the salbutamol molecular structure [16], this compound behaves as a reducing reagent in the reaction system of luminol–hydrogen peroxide. Following administration of salbutamol in humans, it was expected that the CL signal intensity of Hp would be decreased; and this observation could be effective in

Abbreviations: PAGE, polyacrylamide gel electrophoresis; CL, chemiluminescence; Hp, haptoglobin.

* Corresponding authors. Tel.: +86 10 58805373; fax: +86 10 62799838.
E-mail addresses: nana@bnu.edu.cn (N. Na), jinoyang@bnu.edu.cn (J. Ouyang).

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monitoring the changes of salbutamol concentration versus dosing time.

In this study, we report the direct ammonium persulfate-enhanced CL imaging for monitoring the changes of salbutamol concentration in the serum versus dosing time. After PAGE, based on the decrease of CL signal intensity generated from salbutamol, the salbutamol concentration in human serum was predicted. Thus, the present described method will demonstrate its potentials for the individualized dosage regimen and even for pharmaceutical research. We have shown in this study, significant individual difference between the different Hp phenotype groups and the present method indicates good potential for the individualized dosage regimen and for further pharmaceutical research.

2. Materials and methods

2.1. Materials

All reagents were of A.R. grade. Salbutamol and tilidine were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Hp (Hp1-1, Hp 2-2) and tetramethylethylenediamine (TEMED) were obtained from Sigma (St. Louis, MO). Tris-hydroxymethyl aminomethane (Tris) and aminoacetic acid (glycine) were purchased from Sino-American Biotechnology Co. (Beijing, China); N,N′-methylenebisacrylamide (Bis), NaOH, C_2H_5OH, HAc, H_2O_2 and phosphate (NaH_2PO_4, Na_2HPO_4) were obtained from Beijing Chemical Factory (Beijing, China); 3,3′,5,5′-tetrabromophenolsulfonphthalein (Bromophenol Blue) and acrylamide were purchased from Beijing Dingguo Biological Research Institute (Beijing, China). 3-Aminophthalic hydrazide (luminol) was from Acros Organics (NJ), and CBB-R250 was from Fluka (Switzerland). Ammonium persulfate was supplied by Beijing Fine Chemical Factory (Beijing, China). Water was deionized and further purified by a Milli-Q water purification system (Millipore, Milford, MA).

2.2. Instruments

The electrophoresis system consisted of a DYY-6B vertical electrophoresis tank and a DYCZ-24D electrophoresis instrument of steady voltage (Liuyi Instrument Factory, Beijing, China). The developed X-ray films were scanned by a MICROTECK S700 scanner (MICROTECK, China). The fluorescence signal was recorded by RF-5301PC Fluorescence Spectrometer (Shimadzu, Japan).

2.3. Subject selection and sample preparation

Healthy male subjects (age: 18–25 years; weight: 64.2 ± 4.0 kg) were selected as volunteers. The number of oral salbutamol and tilidine volunteers was 18 and 18, respectively. Subjects with a history of hyperthyroidism, diabetes, hypertension, cardiac disease or seizure disorders were excluded. Evidence of any clinically significant abnormalities that might interfere with metabolism and excretion of salbutamol or tilidine was regarded as an exclusion criterion. Subjects were avoided alcoholic drinks and smoking during the study period. All of the subjects gave informed consent to participate in our study, which was expressed agreement with the local ethics committee.

The oral salbutamol and tilidine dose (8 mg) were administered with 250 ml water at morning time, separately. Breakfast was taken 2 h after oral administration. Blood samples were collected through an intravenous catheter 1 h before dose application and at 3, 4, 6, 10, 24 and 48 h after the dose. After careful settlement for 5 h, blood samples were centrifuged at 10,000 rpm for 5 min. Therefore, the

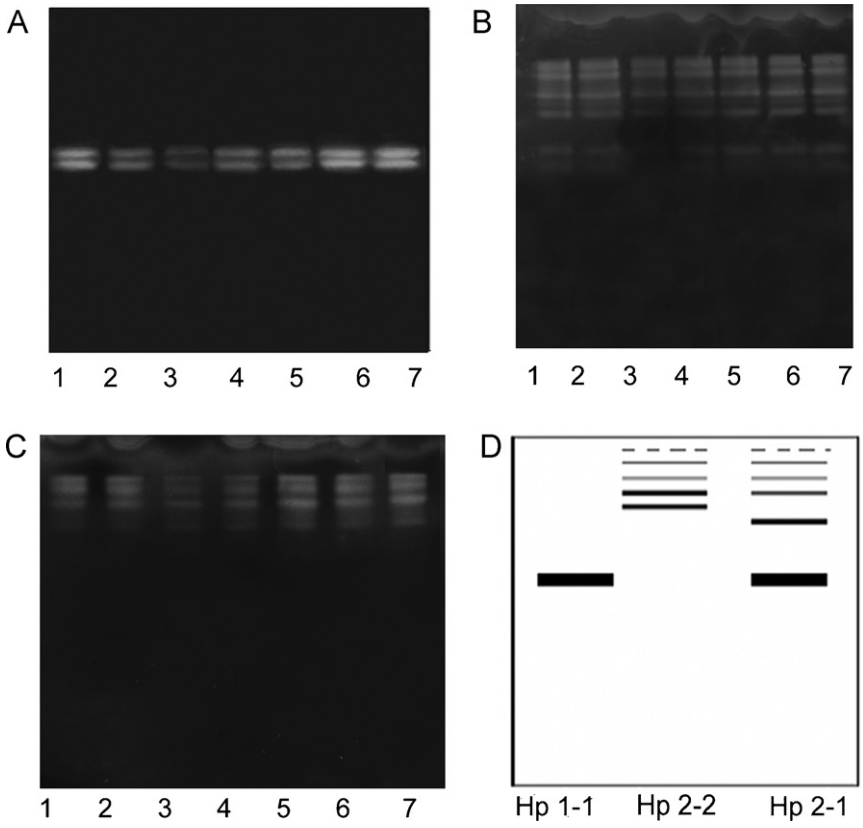

Fig. 1. CL images for the serum from subjects with salbutamol administration. (A) Hp 1-1, (B) Hp 2-1, (C) Hp 2-2. The serum obtained at different time: lane 1, 0 h; lane 2, 3 h; lane 3, 4 h; lane 4, 6 h; lane 5, 10 h; lane 6, 24 h; lane 7, 48 h, (D) standard phenotypes of Hp: Hp 1-1, Hp 2-2 and Hp 2-1.

supernatant was extracted as serum and then stored below −20 °C for further experiment. Serum samples were 10-fold diluted by glycerol solution (20%, v/v) for PAGE, and the loading volume was 15 μl.

2.4. Polyacrylamide gel electrophoresis

The preparation of the no denaturing PAGE was carried out as previously described by Huang et al. [15]. In brief, PAGE was performed in a vertical discontinuous polyacrylamide gel system, composed of 4.0% (m/v) stacking gel with 0.05–0.2% of $(NH_4)_2S_2O_8$, and 7.5% (m/v) separating gel with 0.05% of $(NH_4)_2S_2O_8$. The voltage was set at 120 V in the stacking gel, and then decreased to 90 V for 3 h when the serum sample entered the separating gel. The electrophoresis buffer was Tris–glycine system (pH 8.30). $(NH_4)_2S_2O_8$ solution was at a concentration of 10% (w/v), and daily prepared to ensure the stability.

2.5. CL imaging detection

The polyacrylamide gel was washed with redistilled water three times to avoid the disturbance of primary electrophoresis buffer. It was put on a piece of culture dish, and then the CL detection procedure was carried out in a dark room. After hydrogen peroxide solution (1.2%, v/v) was sprayed onto the gel (about 16 μl/cm^2, for about 15 s), luminol (1.0×10^{-3} M, pH 13) was sprayed onto the gel (about 80 μl/cm^2, for about 1 min) for generating CL. Then with the quick absorption of the solution by filter paper, the gel was covered by a transparency film, after which the X-ray film was exposed for about 2.6 min on the gel. Then, the X-ray film was developed for about 2 min and fixed for about 4 min in order to achieve the CL images. These X-ray films were then scanned, and bands were quantified with a dual-wavelength flying spot scanning densitometer (CS-9301PC, Shimadzu, Japan). Films were scanned with a scanner and the results were transferred to the computer. Luminol stock solution was 0.01 M diluted by NaOH solution (0.1 M).

2.6. Determination of the binding constants

The concentrations of standard Hp 1-1 and Hp 2-2 stock solution were 1.0 mg/ml. 1.0×10^{-3} and 2.0×10^{-3} mg/ml of Hp 1-1 and Hp 2-2 were prepared by diluting the stock solutions in PBS buffer solution (pH 7.40, 20 mM), which were transferred into 5 ml volumetric flasks. Stock solution of standard salbutamol (1.0×10^{-2} M) was prepared by dissolving 0.0024 g of salbutamol into 1 ml water. The appropriate salbutamol concentration was achieved by adding different value of salbutamol stock solution into flasks. Then, the salbutamol–Hp solutions were kept at 37 °C for 2 h. For fluorescence detection, the samples were excited at 280 nm and the fluorescence intensity was monitored at 300–500 nm at 310 K by RF-5301PC Fluorescence Spectrometer. The maximum emission spectrum of the mixture appeared at 320–330 nm.

3. Results and discussion

3.1. Direct CL imaging of sera from subjects with salbutamol administration

In the experiment, sera from 18 healthy male subjects with oral administration of salbutamol were selected as models for the examination. The sera obtained from 0 to 48 h after oral administration of salbutamol were electrophoresed through ammonium persulfate-modified polyacrylamide gels, and the CL images were obtained by the reaction between luminol and hydrogen peroxide.

Hp-related proteins from the subjects with oral administration of salbutamol were recorded by the CL imaging (Fig. 1D shows the standard phenotypes of Hp), which had been confirmed by both standard protein control method [15] and the western-blotting technique [17]. Then, according to the CL images, the samples were separated into three groups based on Hp phenotypes, Hp 1-1, Hp 2-1 and Hp 2-2. As shown in Fig. 1A, different CL signal of protein bands was recorded for sera of Group Hp 1-1, obtained at different dosing time. It was resulted that the CL signal intensity decreased dramatically from 0 to 4 h after oral salbutamol dosing (lane 1 to lane 3), but increased from 6 to 48 h (lane 4 to lane 7). Although more protein bands appeared on the CL images for the groups of Hp 2-1 and Hp 2-2, the lowest CL signal intensity still appeared after 4 h (Fig. 1B and C).

3.2. Changes of salbutamol concentration versus dosing time by direct CL imaging

Therefore, changes of salbutamol concentration versus dosing time for three groups were demonstrated according to the CL imaging. For details, the relative intensity of the imaging representing the total pixel density of the protein bands, which was background intensity subtracted, was measured and exported to Origin 6.0 (Microcal Software, Inc., USA). Fig. 2 shows the curves of relative CL intensity as a function of time for the groups of Hp 1-1, Hp 2-1 and Hp 2-2. As indicated, the CL signal intensity for three groups decreased from 0 to 4 h, and increased to become a plateau region from 4 to 48 h.

The decreased CL signal intensity was generated from the decreased oxidation reaction of the luminol–hydrogen peroxide system with reducing salbutamol. Thus, the curves of CL intensity versus time could be used for monitoring the changes of salbutamol concentration as a function of dosing time in serum. As indicated, the maximum salbutamol concentration in human serum peaked after 4 h, and then started to decrease after four additional hours. This appeared to be caused by either the drug metabolism and/or the binding of the drug to proteins. This was in accordance with the reported profiles achieved by ion chromatography or high performance liquid chromatography with fluorescence detection [18]. Therefore, the use of CL imaging for monitoring changes of salbutamol concentration versus dosing time has further been verified and confirmed.

Although the maximum salbutamol concentration in the serum was observed after 4 h for Hp 1-1, Hp 2-1 and Hp 2-2, the drug concentration kinetics differed between the various groups (as indicated in Fig. 2). For example, after 6 h, the CL intensities of three groups could be ranked as: Hp 1-1 > Hp 2-1 > Hp 2-2. In another word, the salbutamol concentrations in the serum after 6 h were

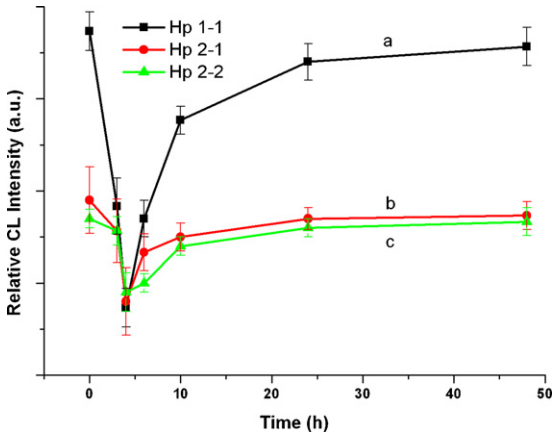

Fig. 2. The relative CL intensity as a function of time for the human serum from the subjects with the oral administration of salbutamol. (a) For the group of Hp 1-1, (b) for the group of Hp 2-1, (c) for the group of Hp 2-2.

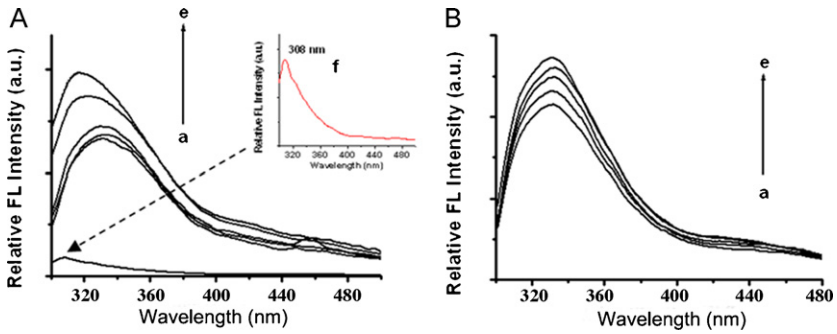

Fig. 3. Fluorescence emission spectra of Hp-salbutamol in PBS buffer pH 7.40, λ_{ex} = 280 nm, temperature = 310 K. (A) For Hp 1-1 (1.0×10^{-6} mg/ml), (f) for pure salbutamol, (B) for Hp 2-2 (2.0×10^{-6} mg/ml). The concentrations of salbutamol were (from a to e): 0, 1.2, 2.4, 3.6, and 4.8×10^{-8} mg/ml, respectively.

ordered as: Hp 1-1 < Hp 2-1 < Hp 2-2. In addition, after the administration of salbutamol for 24 h, the drug concentration was 11.1% of the initial value for Hp1-1, 12.5% for Hp 2-2, but 18.2% for Hp 2-1. Based on this difference, it was concluded that the metabolic rate or the binding ability of salbutamol to Hp 1-1 was much greater than the other two groups. This might be explained by the different binding ability of Hp to salbutamol for different groups. This individual difference recorded by the CL-based imaging method will provide some referential information for the individualized dosage regimen.

3.3. The binding ability of Hp to salbutamol

The study on the interaction of drug with proteins is quite important in pharmaceutical research [19]. Therefore, the binding ability of Hp to salbutamol was examined by fluorescence method with the commercial available standard Hp 1-1 and Hp 2-2 as samples. In the experiment, the fluorescence emission spectroscopy was used to identify the binding constant of Hp 1-1 and Hp 2-2 to salbutamol.

As shown in Fig. 3, when the concentration of salbutamol increased (from 0 to 4.8×10^{-8} mg/ml) within a fixed concentration of Hp, the fluorescence signal intensity at 320–330 nm which was excited at a wavelength of 280 nm increased. In addition, blue shift was resulted only for Group Hp 1-1 with increasing salbutamol concentration. This might generate from the binding of Hp to salbutamol with the emission peak of 308 nm (lower than 320 nm, Fig. 3A-f) [9].

The binding constants of Hp to salbutamol were determined by the fluorescence-based method, considering the following equation as developed by Bhattacharyya, Subramanyam and He et al. [20–22].

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_{max}} + \left(\frac{1}{K[Q]}\right)\left(\frac{1}{\Delta F_{max}}\right) \quad (1)$$

here, $\Delta F = F_x - F_0$ and $F_{max} = F_\infty - F_0$, where F_0, F_x and F_∞ are fluorescence intensities of protein in the absence of drug, at an intermediate concentration of drug, and at the saturation of interaction, respectively. K is the binding constant and [Q] is the drug concentration. Based on the fluorescence signal of Group Hp 1-1 and Group Hp 2-2 with addition of different value of salbutamol (Fig. 3), the linearity in the plot of $1/\Delta F$ against 1/[Q] was obtained, which confirmed a one-to-one interaction between protein and drug. The linearity of $1/\Delta F$ against 1/[Q] for Hp 1-1 (1.0×10^{-8} M) and Hp 2-2 (2.0×10^{-8} M) is shown in Fig. 4. Then, this was followed by calculating the binding constants of Group Hp 1-1 and Group Hp 2-2 to salbutamol based on Eq. (1), which showed that the binding ability of Group Hp 1-1 ($K = 6.31 \times 10^6$ M^{-1}) was higher than Group Hp 2-2 ($K = 2.99 \times 10^6$ M^{-1}). Thus, more salbutamol could bind to Hp 1-1, indicating the greater metabolism rate of salbutamol for Group Hp 1-1, and less salbutamol was remained in serum for Group Hp 1-1. This was in accordance with the greater CL intensity of Hp 1-1 than Group Hp 2-2 after salbutamol administration, which was due to CL quenching ability of salbutamol (Fig. 1).

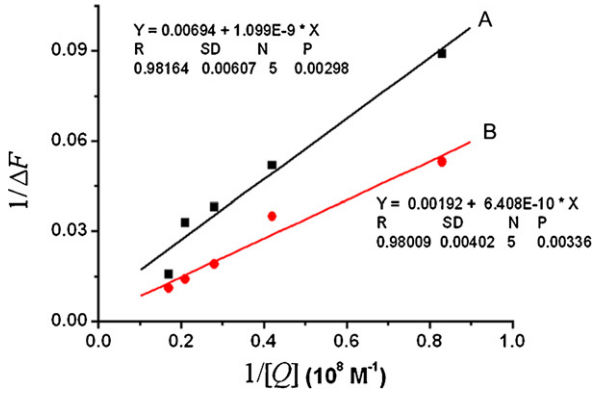

Fig. 4. Plot of $1/\Delta F$ against 1/[Q], where [Q] is the concentration of salbutamol added to Hp 1-1 (A, 1.0×10^{-8} M) and Hp 2-2 (B, 2.0×10^{-8} M). Temperature = 37 °C, pH 7.40.

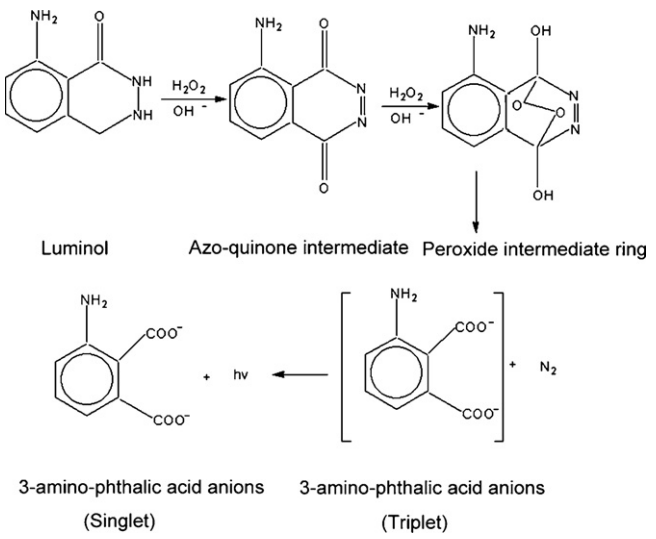

Fig. 5. The mechanism of the reaction between luminol and hydrogen peroxide.

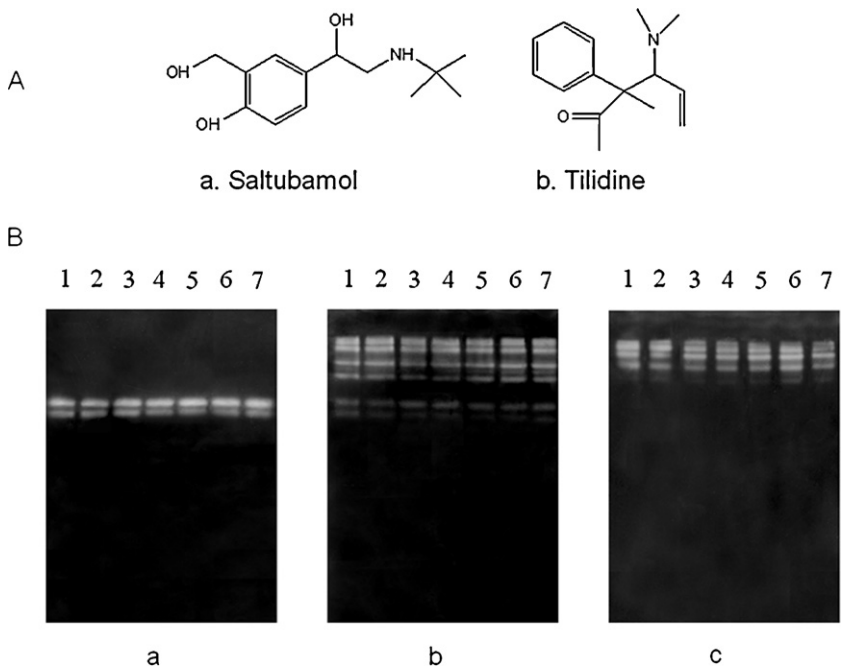

Fig. 6. (A) Structures of salbutamol (a) and tilidine (b), (B) images obtained by CL-imaging for sera from subjects with tilidine administration. (a) Hp 1-1, (b) Hp 2-1, (c) Hp 2-2. The serum obtained at different time: 0 h, 3 h, 4 h, 6 h, 10 h, 24 h and 48 h (from left to right).

3.4. The preliminary study on the possible mechanism

The present CL-based method for monitoring salbutamol concentration in serum was generated from the reducibility of the drug. There was a phenolic hydroxyl group in the molecular structure of salbutamol [16], which resulted its reducibility in the CL generation of luminol–hydrogen peroxide oxidation system (The mechanism of the reaction between luminol and hydrogen peroxide is shown in Fig. 5). CL signal intensity of Hp in luminol–hydrogen peroxide oxidation system decreased due to the presence of reductant salbutamol. Therefore, the decrease of CL intensity could be used for monitoring changes of salbutamol concentration.

To examine the mechanism of CL imaging-based method for monitoring changes of salbutamol concentration, an oxidative drug tilidine (Fig. 6A) was detected for the comparison. In the experiment, sera from subjects in which tilidine had been orally administered were detected by CL imaging after PAGE. Sera were obtained at the time of 0, 3, 4, 6, 10, 24, 48 h after tilidine administration. However, for all three groups, no obvious difference on CL signal intensity was observed among different samples obtained at different time (Fig. 6B), and the curves of relative CL intensity as a function of time still did not show the significant profiles for three groups (Fig. 7). Therefore, the present described CL-based method for monitoring the changes of drug concentration in serum was effective for the drug with reducing ability.

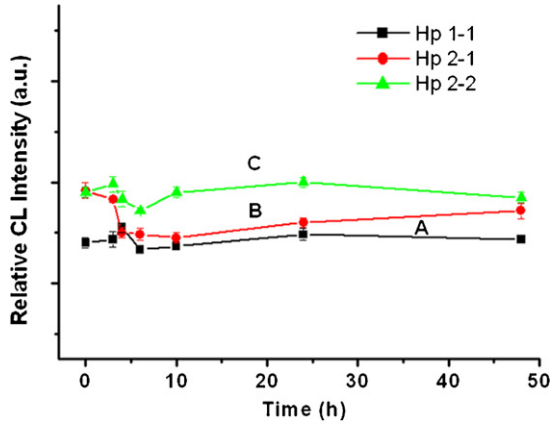

Fig. 7. The relative CL intensity as a function of time for the human serum from the subjects with oral administration of tilidine. (a) For Group Hp 1-1, (b) for Group Hp 2-1, (c) for Group Hp 2-2.

4. Conclusion

In conclusion, the direct ammonium persulfate-enhanced CL imaging after PAGE can be used for monitoring the changes of salbutamol concentration in serum versus time. The described method is based on the reduction of salbutamol. Significant variables between the groups of different Hp phenotypes were also observed and this will supply a reference data for the individualized dosage. This described method omits the separation of salbutamol from the complicated matrix, which makes the detection much simple convenient, and rapid. The present CL-based imaging method shows excellent potential for further use in pharmaceutical research.

Acknowledgements

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