Analytical Methods

PAPER

View Article Online
View Journal | View Issue

Cite this: *Anal. Methods*, 2015, 7, 5635

Investigation of the salbutamol residue level in human urinary samples by a sensitive direct competitive ELISA

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Received 2nd March 2015
Accepted 1st June 2015

DOI: 10.1039/c5ay00544b

www.rsc.org/methods

Salbutamol (SAL) is used to promote animal growth, because it increases feeding efficiency and carcass lean percentage. SAL has been banned in many countries because of the potential hazard to human health. To investigate the SAL residue level in humans, a sensitive direct competitive ELISA was developed. Under the optimal experimental conditions, the limit of detection (LOD) for the assay was 0.01 ng mL^{-1}, and the standard curve was constructed at concentrations of 0.01–100 ng mL^{-1}. We used the developed assay to detect the urinary concentration of SAL in 1648 participants. SAL was detected in 96% of participants with a concentration ranging from the LOD to 30.83 ng mL^{-1}. Pregnant women were found to have a lower SAL concentration than non-pregnant women. Sources of exposure, risk assessment and measures for reducing the exposure level require further investigation.

Introduction

Salbutamol (SAL) is a short-acting β2-adrenergic receptor agonist used in humans for the treatment of obstructive lung disease. Also, it can be used in animal feed to improve an animal's muscle to fat ratio.[1] As a feed supplement, SAL can be accumulated in treated animals and cause a multitude of adverse effects on consumers.[2–4] Therefore, its use has been banned in food-producing animals in many countries including the European Union and China.[5] Since the use of SAL and other analogues in animal feed could lead to double profits, SAL is still used widely and illegally in many farms.[6]

Various techniques and analytical methods have been developed for the measurement of SAL residues in various matrices, including feedstuffs, animal tissues, urine, and so on.[5,7–13] The preferred and official analytical methods for SAL are high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS), with LODs of 18 ng mL^{-1} and 1 ng mL^{-1}. However, these instrumental analysis methods usually require the steps of sample preparation and analysis, and are of high cost and relatively time-consuming. Thus, these methods are used for the confirmatory purpose as a reference analytical method. Nowadays, more and more advanced methods with higher sensitivity have been designed to detect SAL. Electrochemiluminescence (ECL)[14] was developed with an LOD of 17 pg mL^{-1}, and a nanoparticle-biosensor based method[15] was developed with an LOD of 1.44 pg mL^{-1}. These analytical methods are of high cost, operationally complex and usually used in extreme cases for the trace detection of SAL in samples. Enzyme linked immunosorbent assays (ELISAs) appear to be ideal technologies because of the simple sample preparation, low cost, high sensitivity, high specificity and high throughput. Several formats of ELISA have been successfully developed for detecting SAL in animal feed, and the urine and tissue samples of animals.[13,16] However, there are few methods developed that can detect SAL residues in human samples.

Environmental pollution is one of the biggest threats to this planet. Many kinds of natural and synthetic chemical compounds exist in our living environment and pose a great threat to human health.[17–19] Since the detection of human exposure to chemicals is the most direct way to show the level of chemicals in each person, it is very necessary to develop effective methods to detect chemicals in human samples. Like other environmental pollutants, SAL could pose a potential hazard to human health. It is essential to detect the exposure level of humans to SAL. In our present study, we developed a sensitive direct competitive ELISA and performed the assay to detect the exposure to SAL in urinary samples from 1648 participants from China.

Materials and methods

Chemicals and reagents

SAL, SAL–bovine serum albumin conjugate (SAL–BSA) and the antibody against SAL were purchased from Abmart (Shanghai,

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China). Tween-20 and dimethyl sulfoxide (DMSO) were bought from Sinopharm Chemical Reagent (Shanghai, China). 3,3′,5,5′-tetramethylbenzidine (TMB) and horseradish peroxidase (HRP) were bought from Aladdin (Shanghai, China).

Buffers and solutions

The buffers and solutions used in the present experiments are as follows: (1) coating buffer: 0.1 M carbonate buffer (pH 9.6); (2) phosphate-buffered saline (PBS): 10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl [pH 7.4], used for the preparation of standard solutions; (3) washing buffer (PBST): PBS with 0.05% Tween-20 (v/v); (4) blocking buffer: 5% skimmed milk (w/v) in PBST; (5) substrate buffer: 0.1 M citrate (pH 5.5); (6) TMB solution: 6 mg TMB dissolved in 1 mL DMSO; (7) substrate solution: the substrate solution was prepared by adding 125 μL of TMB solution and 2 μL 30% H_2O_2 to 10 mL of substrate buffer. (8) Stop solution: 1 M H_2SO_4. Standard solutions of SAL were prepared from a stock solution of 1 mg mL^{-1} in methanol.

Collection of human urine samples

All urinary samples were collected from 1648 volunteers in the Zhejiang province of China from December 2013–March 2014 with informed consent. Blank urine was obtained from a newborn baby. All samples were centrifuged for 10 minutes, the supernatants were removed to polypropylene tubes and stored at −20 °C. The volunteers, aged from 17–90 years, consisted of a healthy population, diseased population and pregnant population. The diseased population in this study refers to volunteers who have gynecological diseases, liver diseases or cancers. All experiments were performed in compliance with the relevant laws and institutional guidelines, and also have been approved by the local authorities of Zhejiang Province, China.

Preparation of peroxidase tracer (SAL–HRP)

SAL–horseradish peroxidase (SAL–HRP) was synthesized using the periodate method from SAL–BSA and HRP.[20] In brief, 2 mg HRP, dissolved in 0.5 mL deionized water, was reacted with 0.2 mL of 0.1 M sodium periodate and stirred constantly at 37 °C. After 30 min, 0.2 mL of ethylene glycol (1%, v/v) was added to react with the extra sodium periodate. When the ethylene was added, the colour of the reaction solution gradually turned from dark green to brown. Ten minutes later, 1 mg of SAL–BSA, dissolved in 0.5 mL of deionized water, was slowly added to the above brown solution. The mixture was centrifuged and the supernatant was collected, transferred to a dialysis bag and dialyzed against the carbonate buffer (0.05 M, pH 9.6) at 4 °C overnight. The next day, the dialyzed solution was collected, to which was added 0.1 mL of 4 mg mL^{-1} sodium cyanoborohydride. The mixture was reacted at 4 °C for 2 h. Finally, the mixture was dialyzed against phosphate buffer (0.1 M, pH 7.4) at 4 °C for 2 days (three changes of buffer per day). The synthesized SAL–HRP conjugate was mixed with glycerol (1 : 1) and stored in the dark at −20 °C.

Direct competitive ELISA procedure and SAL analysis in urine

For the determination of SAL in human urine, a direct competitive ELISA was developed using a monoclonal antibody against SAL. The present ELISA was performed as described previously with some modifications.[15] Briefly, the wells of a 96-well microplate were coated with 50 μL of antibody solution diluted to a fixed concentration using coating buffer and incubated at 37 °C for 2 h. After washing all wells three times using 200 μL of PBST, the wells were incubated with 100 μL of SAL–HRP diluted to an optimal concentration and 20 μL of the standard samples and/or the urinary samples, the mixtures were incubated at 37 °C for 30 min. The plates were washed three times, 100 μL of substrate solution was then added to each well for the enzyme reaction. 20 min later, 100 μL of stop solution was added to each well to stop the reaction. Finally, the absorbances of all wells were determined at 450 nm by the Bio-Rad microplate reader.

Statistical analysis

All statistical analyses were performed using SPSS 17 (SPSS Inc., Chicago, IL., USA). The concentrations of SAL below the LOD were inferred from the value of the LOD divided by 2. We calculated the geometric mean (GM), minimum, maximum and selected percentiles for the concentration of SAL. The differences between independent groups (gender, pregnant status, and health status) were assessed using the Mann–Whitney U-test. A multiple linear regression model was constructed to examine the correlation of SAL concentration in human urine samples with selected variables (age, gender, pregnant status and health status). The concentrations of SAL were log-transformed before the regression analysis. The statistical significance was set at $P < 0.05$.

Results

Characterization of the direct competitive ELISA

The synthesis of SAL–HRP was identified using a direct ELISA. The results (Fig. 1) showed that SAL was successfully conjugated to HRP. SAL–HRP can be immobilized by the captured antibody and reacted with a substrate to produce a detectable signal. Using SAL–HRP and the antibody against SAL, a direct competitive ELISA was developed to detect the SAL concentration in human urine. The standard curve for the method is presented in Fig. 2. The IC_{50} and LOD were 1.15 ng mL^{-1} and 0.01 ng mL^{-1}. Spiked SAL concentrations (0.1, 1, 10 ng mL^{-1}) in blank urine were used for the validation of the direct competitive ELISA. The recoveries (%) and coefficients of variation (CVs) of SAL in the urinary samples ranged from 86.2% to 112.5% and from 6.23% to 11.52% ($n = 5$), demonstrating the good accuracy and precision of the assay.

Determination of SAL in human urine

Urinary samples were centrifuged to remove precipitates before the measurements using the direct competitive ELISA. Data were corrected for urine dilution by the adjustment of specific gravity. In our present study, the distribution of SAL in all

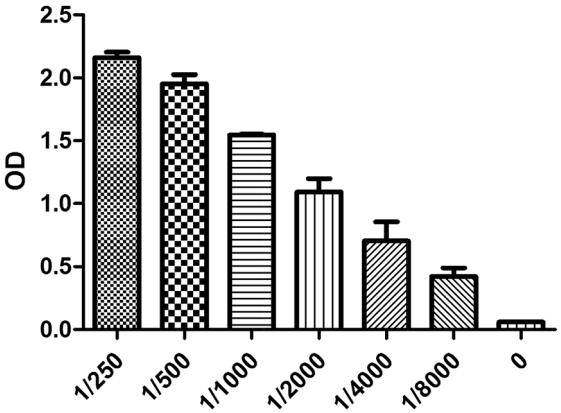

Fig. 1 Identification of SAL–HRP using a direct ELISA. The horizontal axis indicates different dilutions of SAL–HRP. Antibodies against SAL were precoated on the microplate, on which different dilutions of SAL–HRP were then added. After washing the plate, substrate solutions were added to produce a detectable signal.

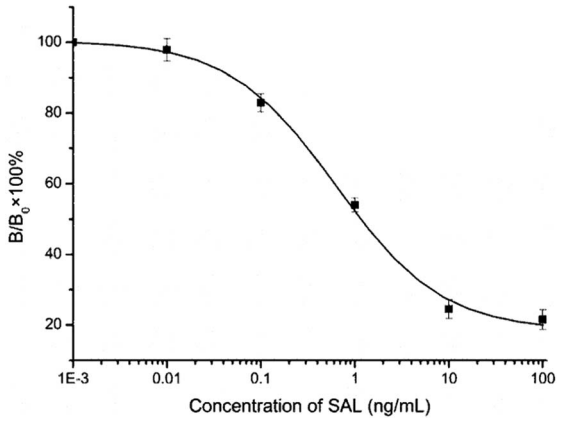

Fig. 2 A representative curve for SAL in a direct competitive ELISA. The vertical bars indicate the standard deviations ($n = 6$).

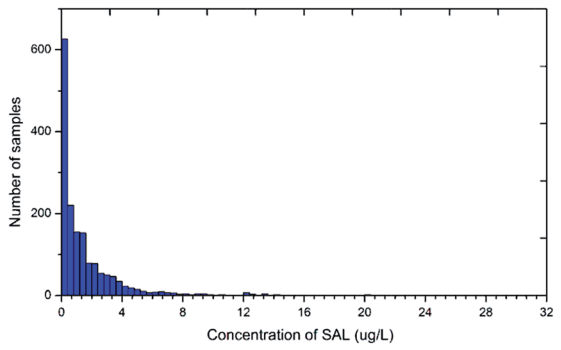

Fig. 3 The distribution of urinary concentration of SAL in all participants. The number of all samples is 1648, and the distribution of SAL in humans is in accord with the characteristic half-normal distribution.

volunteers is shown in Fig. 3 and Table 1. It can be seen that the distribution of SAL is a half-normal distribution, which is a characteristic of the distribution of environmental pollutants in humans. SAL was detected in 96% of participants with a concentration ranging from the LOD to 30.83 ng mL^{-1}. About 32.5% of participants were males, for whom SAL was detected within a range from the LOD to 16.06 ng mL^{-1}, whereas 67.5% of participants were females, for whom SAL was detected within a range from the LOD to 30.83 ng mL^{-1}. In the sample collection process, 571 diseased samples were collected to compare the SAL concentration between a healthy population and a diseased population. SAL was detected in diseased participants within a range from the LOD to 30.83 ng mL^{-1} and in healthy participants within a range from the LOD to 21.18 ng mL^{-1}.

We also distinguished the pregnant women in the female catergory. As shown in Table 2, in pregnant women, SAL was detected within a range from the LOD to 21.18 ng mL^{-1}, and the value of the GM was 0.38 ng mL^{-1}, whereas, in non-pregnant women, SAL was detected within a range from the LOD to 30.83 ng mL^{-1}, and the value of the GM was 0.45 ng mL^{-1}.

In the multiple linear regression model for SAL, we examined the association of selected variables such as age, gender, pregnant status and health status with the log-transformed concentrations of SAL in the urine samples. The results are shown in Table 3. A statistical difference in SAL concentration was found between pregnant women and non-pregnant women. The concentration of SAL in non-pregnant women was significantly higher than that in pregnant women ($P < 0.0001$). No statistically significant difference was found between other groups.

Discussion

In our previous studies, we have developed direct and indirect competitive ELISAs for the detection of environmental pollutants including tartrazine, cotinine, aflatoxin M_1 and bisphenol A.[20–23] All of these assays exhibited good performance in detecting the corresponding chemicals. Meanwhile, direct competitive ELISAs showed better performance in terms of assay time and detection limit. In the present study, we developed a direct competitive ELISA for detecting SAL in human urinary samples. As expected, the developed method can be utilized to assess human samples because of the advantages of high sensitivity, short assay time and high throughput. Using the method developed in this study, we collected 1648 human urinary samples to characterize the exposure of participants to SAL. To the best of our knowledge, we report here for the first time population-based SAL concentrations in humans, so we cannot compare the data with other populations in different periods and/or different countries. Meanwhile, the data can be a reference for future population-based studies to identify exposure trends.

The distribution differences between the different groups were assessed by a multiple linear regression model. The results show that the SAL concentrations are different between pregnant women and non-pregnant women. In females, the GM of the SAL concentrations in pregnant women is lower than that in non-pregnant women ($P < 0.001$). Pregnant women have to pay much more attention to their diet and living environment as they are especially susceptible to the health effects of

Table 1 Selected percentiles for SAL concentration (ng mL^{-1}) in the urine of the Chinese population[a]

				Percentile					
	No.	GM (95% CI)	Min	10th	25th	50th	75th	90th	Max
All	1648	0.50 (0.45–0.55)	<LOD	0.02	0.20	0.73	2.01	3.85	30.83
Gender									
Male	536	0.71 (0.61–0.82)	<LOD	0.04	0.27	1.15	2.64	4.19	16.06
Female	1112	0.42 (0.38–0.47)	<LOD	0.02	0.17	0.59	1.72	3.46	30.83
Age									
17–30	486	0.45 (0.38–0.54)	<LOD	0.02	0.19	0.70	1.86	3.38	20.38
31–40	392	0.62 (0.51–0.74)	<LOD	0.02	0.26	0.87	2.54	4.68	21.18
41–50	358	0.49 (0.41–0.60)	<LOD	<LOD	0.24	0.72	1.94	3.71	30.83
51–60	156	0.47 (0.36–0.62)	<LOD	0.03	0.17	0.62	1.82	3.23	13.47
61–70	153	0.43 (0.32–0.59)	<LOD	<LOD	0.13	0.70	1.82	3.84	20.28
71+	103	0.50 (0.34–0.72)	<LOD	0.03	0.11	0.69	2.34	4.66	16.06
Health status									
Diseased	571	0.42 (0.36–0.49)	<LOD	<LOD	0.19	0.59	1.54	3.35	30.83
Healthy	1077	0.55 (0.49–0.61)	<LOD	0.02	0.21	0.87	2.29	4.02	21.18

[a] GM: geometric mean.

environmental contaminants. The data in our study indeed show that pregnant women have a lower SAL concentration. Since the metabolisms and lifestyles of pregnant women are quite different from those of non-pregnant women, it is hard to determine the causes that contribute to the lower SAL concentrations.

Many published reviews and epidemic investigation results show that the exposure of humans to environmental pollutants poses a great threat to human health.[24–26] The "safe level" of environmental pollutants in human beings and the association between environmental pollutants and human diseases have been the emphasis and difficulty in the field of human health. Like other environmental pollutants, researchers have not found the exact relationship between SAL and specific diseases. So, we collected 571 samples from volunteers with common diseases as our disease samples to use in a preliminary study of the relationship between these diseases and SAL concentration in human beings. However, the data in our present study show that no statistically significant difference is found between the diseased population and the healthy population. Since assessment of the relationship between exposure to environmental pollutants and disease is complicated,[27,28] the results cannot deny the association between exposure to SAL and potential adverse health. There is much we should study about the exposure to environmental pollutants and the adverse effects, in order to determine the "safe level" of SAL in the human body.

Conclusions

In this study, we developed a direct competitive ELISA for detecting SAL. This method has the potential to detect SAL in human samples because of its simple sample preparation, low cost, high sensitivity and high throughput. To investigate the SAL residue level in humans, we applied the present method to detect the urinary SAL concentrations in 1648 participants. The distributions and descriptive statistics of SAL concentrations in different populations were analyzed by statistical software. Human data indicates that many people have realized the potential threat of environmental pollutants and that human exposure to environmental pollutants could be reduced by appropriate measures.

Table 2 Selected percentiles for SAL concentration (ng mL^{-1}) in the urine of the females

				Percentile					
	No.	GM (95% CI)	Min	10th	25th	50th	75th	90th	Max
Pregnant	480	0.38 (0.32–0.45)	0.01	0.02	0.05	0.62	1.64	3.62	21.18
Diseased	182	0.37 (0.27–0.50)	0.01	0.01	0.05	0.58	1.77	3.59	20.38
Healthy	298	0.38 (0.30–0.48)	0.01	0.02	0.05	0.63	1.55	3.81	21.18
Non-pregnant	632	0.45 (0.39–0.52)	0.01	0.02	0.20	0.57	1.74	3.34	30.83
Diseased	351	0.43 (0.35–0.52)	0.01	0.01	0.23	0.54	1.45	3.26	30.83
Healthy	281	0.49 (0.40–0.61)	0.01	0.02	0.17	0.59	2.22	3.36	10.67

Table 3 Regression model[a] for urinary SAL (ng mL^{-1}, natural log-transformed) of participants in this study

Variable	Exponential coefficient, β (95% CI)	*P*-value
Constant	0.845 (0.152–1.539)	0.017
Age	0.003 (−0.008–0.014)	0.582
Gender	0.042 (−0.459–0.542)	0.870
Pregnant status		
Non-pregnant women *vs.* pregnant women	1.605 (1.209–2.002)	<0.001
Health status		
Healthy *vs.* diseased	−0.009 (−0.150–0.132)	0.902

[a] Each variable was adjusted for the others in the model, r^2 and r^2 adjusted for the model are 0.237 and 0.231, respectively.

Acknowledgements

This project was supported by the National Science and Technology Major Project for "Major New Drug Project" [Grant no. 2012ZX09506001-004].

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