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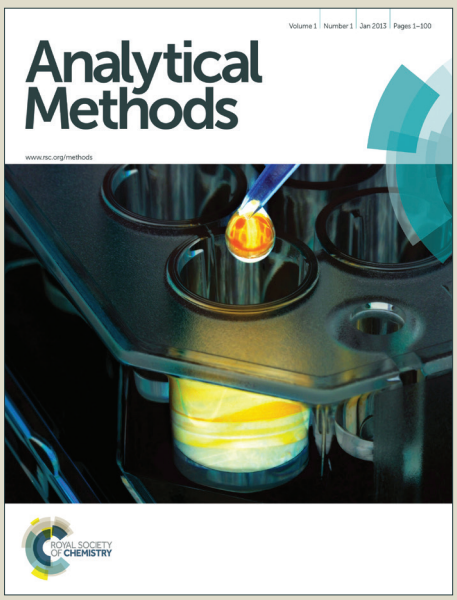

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ARTICLE TYPE

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

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Salbutamol (SAL) was used to promote animal growth, increase feeding efficiency and carcass lean percentage as a growth promoter. SAL has been banned in many countries because of the potential hazard to human health. To investigate the SAL residue level in human, 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, the standard curve was constructed at concentrations of 0.01-100 ng/mL. We performed the developed assay to detect the urinary concentration of SAL in 1648 participants. SAL was detected in 96% of participants with concentration ranging from LOD to 30.83 ng/mL. Pregnant women were found to have lower SAL concentration than non-pregnant women. Sources of exposure, risk assessment and measures how to reduce the exposure level require further investigation.

Keywords: Salbutamol, ELISA, human exposure, pregnant

Introduction

Salbutamol (SAL) is a short-acting β2-adrenergic receptor agonist used in human for the treatment of obstructive lung disease. Also, it can be used in animal feed to improve the animal's muscle to fat ratio [1]. As a feed supplement, SAL can be accumulated in animal with treated and cause a multitude of adverse effects on consumers [2-4]. Therefore, it has been banned to be used on food-producing animals in many countries including 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 feedstuff, 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 the LODs of 18 ng/mL and 1 ng/mL. However, these instrumental analysis methods usually require the step of sample preparation and analysis, and are of high cost and relatively time-consuming. Thus, these methods are used for the confirmatory purposed as a reference analytical method. Nowadays, more and more advanced methods with higher sensitivity were designed to detect SAL. Electrochemiluminescent (ECL) [14] was developed with the LOD of 17 pg/mL, nanoparticles-biosensor based method [15] was developed with the LOD of 1.44 pg/mL, these analytical methods are of high cost, complex operation and usually used for the extremely trace detection in a litter samples. Enzyme linked immunosorbent assays (ELISAs) appear to be ideal technologies because of simple samples preparation, low cost, high sensitivity, high specific and high through put. Several formats of ELISA have been successfully developed for detecting SAL in animal feed, and urine and tissue samples of animals [13, 16]. However, there are few methods were developed to detect SAL residues in human samples.

Environmental pollution is one of the biggest threats to this planet. 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 post potential hazard to human health. It is inevitable to detect the exposure level of human to SAL. In our present study, we developed a sensitive direct competitive ELISA and performed the assay to detect the exposure to SAL using urinary samples from 1648 participants of China.

Materials and methods

Chemicals and reagents

SAL, SAL-bovine serum albumin conjugate (SAL-BSA) and

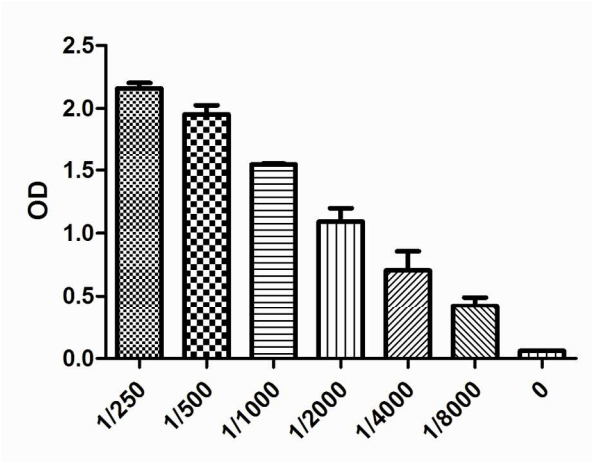

Fig. 1 Identification of SAL-HRP by direct competitive ELISA. Horizontal axis indicates different dilutions of SAL-HRP. Antibodies against SAL were precoated on the microplate, on which different dilutions of SAL-HRP were then added. After washing the plate, substrate solution were added to produce the detection signal.

antibody against SAL were purchased from Abmart (Shanghai, China). HRP was bought from Aladdin (Shanghai, 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). Horseradish peroxidase labelled goat anti-rabbit lgG conjugate (HRP-GaR lgG) was purchased from Boshide (Wuhan, China).

Buffers and solutions

Buffers and solutions used in the present experiments are shown as follows: (1) coating buffer: 0.1M carbonate buffer (pH9.6); (2) Phosphate-buffered saline (PBS): 10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl [pH 7.4], used for preparation of standard solution; (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: 0.1M citrate (pH 5.5); (6) TMB solution: 6 mg TMB dissolved in 1 mL DMSO; (7) substrate solution: 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: 1M H_2SO_4. Standard solutions of SAL were prepared from a stock solution of 1 mg/mL in methanol.

Collection of human urine samples

All urinary samples were collected from 1648 volunteers in 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 ℃. The volunteers, aged from 17-90 years, contained healthy population, diseased population and pregnant population. Diseased population in this study refer to the 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-horse reddish peroxidase (SAL-HRP) was synthesized by the periodate method using SAL-BSA and HRP [20]. In briefly, 2 mg HRP, dissolved in 0.5 mL deionized water, was reacted with 0.2 mL 0.1 M sodium periodate and stirred constantly at 37 ℃. After 30 min, 0.2 mL ethylene glycol (1%, v/v) was added to react with the extra sodium periodate. When the ethylene was added, the colour of reaction solution was gradually turned from dark green to brown. Ten minutes later, 1 mg SAL-BSA, dissolved in 0.5 mL deionized water, was slowly added to the above brown solution. Centrifuged the mixture and collected the supernatant, which was transferred to a dialysis bag and dialyzed against the carbonate buffer (0.05 M, pH 9.6) at 4 ℃ overnight. The next day, collected the dialyzed solution, to which added 0.1 mL of 4 mg/mL sodium cyanoborohydride. The mixture was reacted at 4 ℃ for 2 h. Finally, the mixture was dialyzed against phosphate buffer (0.1 M, pH7.4) at 4 ℃ 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 ℃.

Direct competitive ELISA procedure and SAL analysis in urine

For the determination of SAL in human urine, a direct competitive ELISA was developed using monoclonal antibody against SAL. The present ELISA was performed as described previously with some modifications [15]. Briefly, the well of 96-well microplates were coated with 50 µL of antibody diluted in a fixed concentration using coating buffer and incubated at 37 ℃ for 2 h. After washing all wells three times using 200 µL PBST, the wells were incubated with 100 µL SAL-HRP diluted at a optimal concentration and 20 µL standard samples and/or urinary samples, the mixtures were incubate at 37 ℃ 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 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.

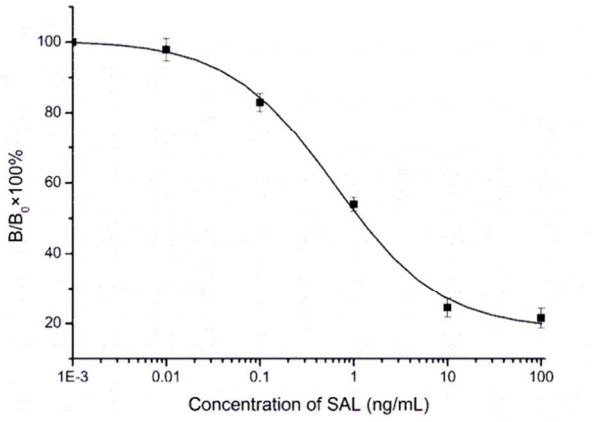

Fig. 2 Representative curve for SAL in direct competitively ELISA. Vertical bars indicate standard deviations (n=6).

Statistical analysis

All statistical analyses were performed using SPSS 17 (SPSS Inc., Chiago, IL., USA). The concentrations of SAL below the

LOD were imputed by the value of LOD divided by 2. We calculated geometric mean (GM), minimum, maximum and selected percentiles for the concentration of SAL. The differences between

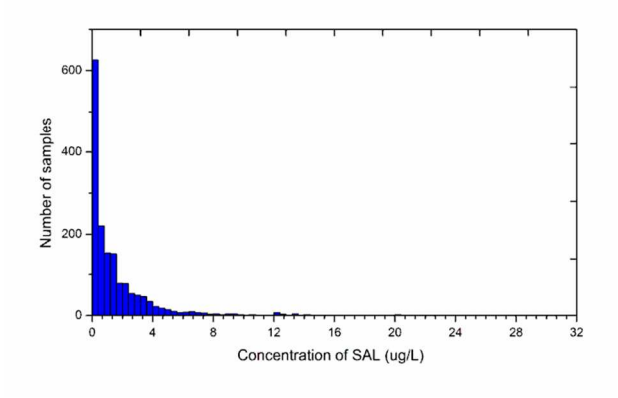

Fig. 3 Distribution of urinary concentration of SAL in all participants. Number of all samples is 1648, and the distribution of SAL in human accord with the characteristic of half-normal distribution.

independent groups (gender, pregnant status, and health status) were assessed using the Mann-Whitney U-test. Multiple linear regression model was constructed to examine the correlation of SAL concentrations 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. Statistical significance was set at $P < 0.05$.

Results

Characterization of direct competitive ELISA

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

Table 1 Selected percentiles of SAL concentration (ng/mL) in urine for the Chinese population.

	No.	GM（95%CI）	Min	Percentile					Max
				10th	25th	50th	75th	90th	
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 statue									
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

GM: Geometric mean.

Determination of SAL in human urine

Urinary samples were centrifuged to remove precipitate before the measurement by 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 volunteers is shown in Figure 3 and Table 1. It can be seen that the distribution of SAL is half-normal distribution, which is a characteristic for the distribution of environmental pollutants in human. SAL was detected in 96% of participants with concentration ranging from LOD to 30.83 ng/mL. About 32.5% participants were males, who were detected with the range from LOD to 16.06 ng/mL, whereas 67.5% participants were females, who were detected with the range from LOD to 30.83 ng/mL. In the sample collection process, 571 diseased samples were collected to compare the SAL concentration between healthy population and diseased population. SAL was detected in diseased participants with the range from LOD to 30.83 ng/mL and in healthy participants with the range from LOD to 21.18 ng/mL.

We also distinguished the pregnant women from females. As shown in table 2, in pregnant women, SAL was detected with the range from LOD to 21.18 ng/mL, the value of GM was 0.38 ng/mL, whereas, in non-pregnant women, SAL was detected with the range from LOD to 30.83 ng/mL, the value of GM was 0.45 ng/mL.

Table 2 Selected percentiles of SAL concentration (ng/mL) in urine for the females.

	No.	GM（95%CI）	Min	Percentile					Max
				10th	25th	50th	75th	90th	
Pregnant	480	0.38 (0.32-0.45)	0.01	0.02	0.05	0.62	1.64	3.62	21.18
Disease	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
Disease	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 (μg L^{-1}, natral log-transformed) from 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		
Nonregnant women vs. pregnant women	1.605 (1.209-2.002)	< 0.001
Health status		
Health 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.

In the multiple linear regression model for SAL, we examine the association of selected variables such as age, gender, pregnant status and health status with the log-transformed concentrations of SAL in urine samples. The results were shown in table 3. 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, 21-23]. All of these assays exhibited good performance in detecting corresponding chemicals. Meanwhile, direct competitive ELISA showed better performance in consideration 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 detect human samples because of the advantage of high sensitive, 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 human, 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 different groups were assessed by multiple linear regression model. The results show that the SAL concentrations are different between pregnant women and non-pregnant women. In the females, the GM of 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 environmental contaminants. The data in our study indeed show that pregnant women have lower SAL concentration. Since the metabolism and lifestyle in pregnant women are quite different from nonpregnant women, it is hard to conclude the causes that contribute to the lower SAL concentration.

Many published reviews and epidemic investigation results show that exposure of human to environmental pollutants post a great threaten 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 of the field of human health. Like other environmental pollutants, researchers have not found the exact relationship between the SAL and specific disease. So we collected 571 samples with common diseases as our disease samples to preliminary study the relationship of these diseases and SAL concentration in human beings. However, the data in our present study show that no statistically significant different is found between diseased population and 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 adverse effects, and then find out the "safe level" of SAL in human body.

Conclusions

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

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References

1. Yamini, Y., et al., Extraction and preconcentration of salbutamol and terbutaline from aqueous samples using hollow fiber supported liquid membrane containing anionic carrier. Journal of Chromatography A, 2006. 1124(1): p. 57-67.
2. Khamta, Y., et al., Development of immunochromatographic assay for the on-site detection of salbutamol. Journal of Immunoassay and Immunochemistry, 2009. 30(4): p. 441-456.
3. Sheu, S.-Y., et al., Screening of salbutamol residues in swine meat and animal feed by an enzyme immunoassay in Taiwan. Analytica chimica acta, 2009. 654(2): p. 148-153.
4. Wang, J.P. and J.Z. Shen, Immunoaffinity chromatography for purification of Salbutamol and Clenbuterol followed screening and confirmation by ELISA and GC-MS. Food and agricultural immunology, 2007. 18(2): p. 107-115.
5. Xie, C.-h., F.-j. Chen, and T.-b. Yang, A high-affinity anti-salbutamol monoclonal antibody: Key to a robust lateral-flow immunochromatographic assay. Analytical Biochemistry, 2012. 426(2): p. 118-125.
6. Kearns, C.F., et al., Chronic administration of therapeutic levels of clenbuterol acts as a repartitioning agent. Journal of Applied Physiology, 2001. 91(5): p. 2064-2070.
7. Garrido, B.C., et al., Salbutamol extraction from urine and its stability in different solutions: identification of methylation products and validation of a quantitative analytical method. Biomedical Chromatography, 2013. 27(12): p. 1630-1638.
8. Rosales‐Conrado, N., et al., Determination of salbutamol by direct chiral reversed‐phase HPLC using teicoplanin as stationary phase and its application to natural water analysis. Biomedical Chromatography, 2013.
9. Traynor, I., et al., Detection of multi-β-agonist residues in liver matrix by use of a surface plasma resonance biosensor. Analytica chimica acta, 2003. 483(1): p. 187-191.
10. Yan, Z., et al., A label-free immunosensor for determination of salbutamol based on localized surface plasmon resonance biosensing. Bioprocess and biosystems engineering, 2013: p. 1-7.
11. Zhang, D., et al., Determination of salbutamol in human plasma and urine using liquid chromatography coupled to tandem mass spectrometry and its pharmacokinetic study. Biomedical Chromatography, 2012. 26(10): p. 1176-1182.
12. Zhang, J., et al., Quantitation of salbutamol in human urine by liquid chromatography–electrospray ionization mass spectrometry. Journal of Chromatography B, 2006. 831(1): p. 328-332.
13. Zuo, P., et al., Determination of beta-adrenergic agonists by hapten microarray. Talanta, 2010. 82(1): p. 61-6.
14. Li, Z., et al., Highly sensitive near-simultaneous assay of multiple "lean meat agent" residues in swine urine using a disposable electrochemiluminescent immunosensors array. Biosens Bioelectron, 2013. 39(1): p. 311-4.
15. Wang, H., et al., A silver-palladium alloy nanoparticle-based electrochemical biosensor for simultaneous detection of ractopamine, clenbuterol and salbutamol. Biosens Bioelectron, 2013. 49: p. 14-9.
16. Pleadin, J., et al., Rapid immunoassay method for the determination of clenbuterol and salbutamol in blood. J Anal Toxicol, 2013. 37(4): p. 241-5.
17. Delfosse, V., et al., A structural perspective on nuclear receptors as targets of environmental compounds. Acta Pharmacol Sin, 2014.
18. Steinle, S., et al., Personal exposure monitoring of PM2.5 in indoor and outdoor microenvironments. Sci Total Environ, 2015. 508: p. 383-94.
19. Hashim, D. and P. Boffetta, Occupational and Environmental Exposures and Cancers in Developing Countries. Ann Glob Health, 2014. 80(5): p. 393-411.
20. Lei, Y., et al., Development and comparison of two competitive ELISAs for the detection of bisphenol A in human urine. Analytical Methods, 2013. 5(21): p. 6106-6113.
21. Lei, Y., et al., Estimation of Urinary Concentration of Aflatoxin M1 in Chinese Pregnant Women. Journal of food science, 2013. 78(11): p. T1835-T1838.
22. Lei, Y., et al., A sensitive and specific enzyme immunoassay for detecting tartrazine in human urinary samples. Analytical Methods, 2013. 5(4): p. 925-930.
23. Lei, Y., et al., Development and comparison of two competitive ELISAs for estimation of cotinine in human exposed to environmental tobacco smoke. Drug Test Anal, 2014. 6(10): p. 1020-7.
24. Lang, I.A., et al., Association of urinary bisphenol A concentration with medical disorders and laboratory abnormalities in adults. JAMA, 2008. 300(11): p. 1303-10.
25. Gluckman, P.D. and M.A. Hanson, Living with the past: evolution, development, and patterns of disease. Science, 2004. 305(5691): p. 1733-6.
26. Stillerman, K.P., et al., Environmental exposures and adverse pregnancy outcomes: a review of the science. Reprod Sci, 2008. 15(7): p. 631-50.
27. Clinkenbeard, R.E., et al., A field comparison of the IOM inhalable aerosol sampler and a modified 37-mm cassette. Appl Occup Environ Hyg, 2002. 17(9): p. 622-7.
28. Koppe, J.G., et al., Exposure to multiple environmental agents and their effect. Acta Paediatr Suppl, 2006. 95(453): p. 106-13.