P70: TAILOR MADE SORBENT FOR SOLID PHASE EXTRACTION OF SALBUTAMOL IN POULTRY MEAT AND DETECTION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract – A highly selective molecularly imprinted polymer (MIP) was synthesized by precipitation polymerization and was used as sorbent material for solid phase extraction (SPE) of salbutamol (SAL) in poultry meat. The MIPs' performance for the selective extraction of SAL in meat was optimized and compared using commercial SPE. A linear calibration curve was obtained with R > 0.99 for HPLC. Recoveries of SAL using HPLC for meat spiked samples were greater than 90 %. The LOD and LOQ obtained by HPLC were 0.08 µg/g and 0.25 µg/g, respectively.

Keywords: Molecular imprinted polymer, solid phase extraction and salbutamol

1. INTRODUCTION

Nowadays, food safety issues have been the focus of concern of the government and consumers worldwide due to large number of cases of chemical contamination in food. One such event was the β_2-agonists intoxication incident in human after eating contaminated meat in countries like China, Mexico, and Portugal [1].

Salbutamol (SAL), [2-(tert-butylamino)-1-(4-hydroxy-3(hydroxylmethyl)phenyl)ethanol] is the most commonly used β_2-agonists for medication for the treatment of bronchial diseases, asthma and other allergic diseases related with respiratory pathway [2]. However, it is used illegally as a growth-promoting agent (also known as "lean meat agents") to increase the carcass leanness and feeding efficiency, and to reduce fat deposition in various food-producing animals [3]. The European Union members, China and some other countries, as well as the Philippines, have banned using β_2-agonists as growth-promoting agents in animals [4].

In the Philippines, the National Meat Inspection Service (NMIS) performs the monitoring and surveillance of drug residues in livestock and poultry meat, however, they only monitor β-agonist drugs in hog and cattle. Chicken meat sample is not checked due to its non-industrial and technological advantage [5]. Thus, there is a need for a simple, fast and cheap method specific to SAL in chicken meat samples.

Recently, solid phase extraction based on molecularly imprinted polymers (MISPE) is gaining significant interest in analysis of food, clinical and environmental samples. Molecular imprinted polymer (MIP) possesses complementary binding sites for the specific recognition of the target molecule and can be used as a sorbent in solid phase extraction of SAL in food samples [6]. Thus, by preparing a MISPE for SAL, sample clean-up could be easier and faster. This study aimed to synthesize and characterize a SAL-molecularly imprinted polymer for use as sorbent material for solid phase extraction in SAL prior to HPLC analysis.

2. EXPERIMENTAL

2.1 Reagents and standards

Salbutamol (99 %) and clenbuterol (95 %) standards from Sigma, methacrylic acid (MAA), trimethylolpropane trimethacrylate (TRIM) and 2, 2'-azoisobutyronitrile (AIBN) from Sigma-Aldrich were used in this work. HPLC grade and analytical grade solvents, analytical grade orthophosporic acid, and ammonia (UNIVAR) and potassium dihydrogen phosphate (Merck) were used in the experiments.

2.2 Instrumentation

High Performance Liquid Chromatography (HPLC) (Shimadzu Prominence LC-20A) system equipped with LC-20AT Solvent delivery unit, SIL-20AC HT Autosampler, SPD-M20A Diode Array Detector, CBM-20A Communication Bus Module, Sunfire™ C18 (3.5 µm, 150 mm × 4.6 mm I.D.) column, and LCSolution workstation was applied for analysis. The mobile

phase (0.5 mL/min) was 0.05 M potassium dihydrogen phosphate buffer (pH 3.0)-acetonitrile (90:10, v/v). The mobile phase was also used as the diluent for the standard solutions and sample. The injection volume used was 20 µL. The UV detection wavelength for SAL was 225 nm. External calibration curve was used for quantitation.

2.3 MIP prepared by Precipitation polymerization

One mmol of SAL and 4 mmol of MAA was dissolved in 50.0 mL dichloromethane in a 250 mL media bottle. To the mixture 4 mmol of TRIM and 1 mmol of AIBN were added. This was sonicated (5 min) and then purged with nitrogen gas for 5 minutes. Polymerization (24 hr) was done in a shaking water bath set at 60°C, 120 cycles/min. The polymer particles was sieved using 38 µm mesh (Fisher Scientific Company). Soxhlet extraction of the MIP was conducted using methanol-acetic acid (90:10, v/v) solvent mixture for 50-60 hours. After extraction, the obtained polymers were rinsed with acetone to remove the remaining methanol-acetic acid mixture and dried. Non imprinted polymer (NIP) which served as a control, was also prepared with similar conditions but without the addition of the template SAL.

2.4 Sample preparation

Fresh organic chicken meat was purchased from Supreme Harvest Farm and was used for method development. The fresh breast and thigh parts of the chicken meat samples were homogenized separately using Retsch Knife Mill. For the recovery analysis, 25 grams of the blank chicken meat sample was spiked with 0.3 and 0.6 µg/g of SAL, respectively. Then, it was stored overnight in freezer before analysis. The sample (0.1 g) was extracted twice with 0.5 mL ACN: NH_3 (95:5, v/v) for 10 minutes under ultrasonic vibration. The supernatant was concentrated to dryness using N_2 gas and was reconstituted with 1.5 mL of distilled water for further MISPE procedure.

2.5 MISPE conditions

The cartridge was pre-conditioned with 2.0 mL methanol, followed by 1.0 mL of water. The cartridge was then loaded with 1.0 mL of the standard SAL or sample solution for the MISPE process. After loading the sample solutions, the cartridge was washed with 1 mL of methanol: water (30:70, v/v), and then eluted with 1 mL methanol: acetic acid (95:5, v/v). The collected eluent was evaporated to dryness using nitrogen gas. The residue was reconstituted in 1.0 mL mobile phase solution for further HPLC analysis.

3. RESULTS AND DISCUSSION

3.1 Synthesis of MIP

In this study, an MIP using SAL as template was prepared by precipitation polymerization using methacrylic acid (MAA) as functional monomer, trimethylolpropane trimethacrylate (TRIM) as the cross-linker, 2,2′-azobis(2-methylpropionitrile) (AIBN) as the initiator, and dichloromethane (DCM) as the porogen. During the MIP synthesis, the functional monomers (MAA) self-assembled around the SAL template molecule, through non-covalent interactions. It formed a "template-monomer complex" that polymerized by the application of heat in the presence of the cross-linker (TRIM) and initiator (AIBN). After polymerization, the SAL template was extracted from the obtained polymer leaving behind specific cavities complementary to shape, size and functionalities of SAL. The percent (%) yield achieved for MIP and NIP were about 90.8 % and 72.9 %, respectively.

3.2 Optimization of the MISPE process

MIP and NIP particles were packed in polypropylene cartridges and were used on off-line mode. The applicability of the MIPs for extraction and separation of trace level of SAL from complex samples was evaluated by optimizing the MISPE solvents for loading, washing and elution.

The SPE cartridge was pre-conditioned using methanol, followed by water. Then different loading solvents, such as methanol, water and dichloromethane were used to load 5 µg/mL SAL in the prepared MIP and NIP. DCM gave the lowest recovery which implied that SAL was retained in the MIP, however slow elution rate was observed, and this might be due to the strong halogen bonding of DCM to the MIP. On the other hand, using water and methanol, as loading solvent for MIP, the percent SAL recovery was 0.30 % and 3.63 %, respectively. Since the percent SAL recovery was low using water, it was used as the loading solvent. Comparing the MIP and NIP as sorbents, MIP showed a lower recovery of SAL (0.30 %) than NIP (6.88%), depicting MIPs better retention for SAL.

The most critical step in SPE protocol was the washing step. This step maximizes the special interactions between the analyte and binding sites where matrix interferences could be removed in the SPE without losing the analyte. One mL of different washing solvents such as methanol-water (50:50, v/v),

acetonitrile, acetonitrile-water (50:50, v/v), methanol, acetone-chloroform (50:50, v/v) and dichloromethane were tried to remove interferences. Using non-polar solvents such as acetone-chloroform and DCM resulted to a slow elution rate of the solvents thus analysis using non polar solvents were discontinued.

Methanol-water (50:50, v/v) mixture was selected as the washing solvent because it gave the lowest recovery of SAL (12.9%) in the washing step and was sufficient to elute cleaner extract (SAL recovery =74.8 %) using methanol-acetic acid (90:10, v/v) as elution solvent. The effect of different ratio of methanol-water (70:30, 50:50 and 30:70, v/v) were further analyzed to increase its efficiency. Using methanol-water (30:70, v/v) resulted to lowest recovery (2.3%), thus the optimized washing solvent was 1.0 mL of this solution.

After the washing process, strong interaction between the imprint and the analyte should be destroyed in order to have high extraction recovery. A polar solvent containing an acidic or basic component can effectively interrupt the hydrogen bond interaction of the template to the MIP binding sites. Elution of 5 µg/mL SAL was analyzed using 1 mL of different solvent mixtures such as methanol-acetic acid (90:10, 95:5 and 99:1, v/v), methanol-ammonia (90:10 and 95:5, v/v), acetonitrile-acetic acid (90:10, v/v), acetone-acetic acid (90:10, v/v), water-acetic acid (90:10, v/v) and pure methanol. Methanol-acetic acid (95:5, v/v) showed the highest recovery of SAL which was 93.5 % and has good precision results (Fig. 1). Thus, adding 5% of weak acetic acid destroys the strong interaction and restrained the non-selective binding between SAL an MIP, thus obtaining good recoveries.

Volume consumption (1-3 mL) of the elution solvent was also optimized to minimize solvent consumption. Results showed that after 1.0 mL, no more SAL traces were determined from the extraction solvent. Therefore, considering efficiency and solvent consumption of the elution solvent, 1.0 mL of methanol-acetic acid (95:5, v/v) was the optimum elution solvent.

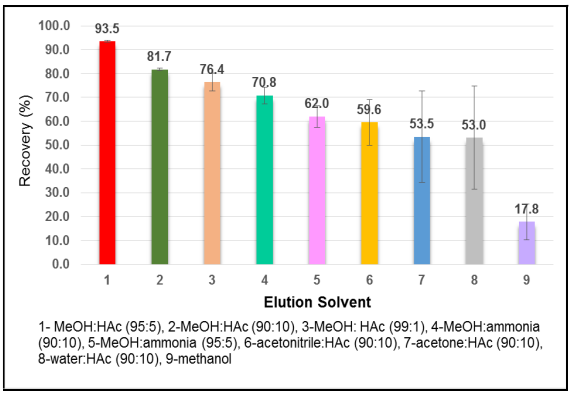

Figure 1. Recoveries of SAL in elution fractions with different solvents after SPE of 5 µg/mL SAL standard solution on 20 mg polymer (n=2)

To evaluate the capacity of the polymers to retain SAL in the prepared MISPE cartridge, varying amount of packed polymer using 5.0, 10.0, 15.0, 20.0 and 25.0 mg and loading single concentration of SAL standard in water (50 µg/mL) were considered. SAL recovery (98.0 %) was highest when 20 mg polymer was used as sorbent.

Elution characteristics of the synthesized polymer (20 mg) packed in cartridges were compared with commercial SPE cartridge such as Supelco Supelclean™ LC-18 SPE tube (C18, 100 mg, 20 µm porosity) and Merck LiChrolut® RP-18 (C18, 500 mg, 40-63 µm porosity). The MIP showed a higher recovery (97.1 %) after loading 5 µg/mL SAL as compared to using Supelco and Merck SPE tubes which eluted 25.2 % and 66.8 %, SAL respectively. This could be due to nonpolar and non-specific interactions of SAL with the commercial C18 SPEs.

To study the selectivity of the prepared MISPE, another β-agonist (clenbuterol) that is illegally used as a growth promoting agent was used. Mixed solution of SAL and clenbuterol (10 µg/mL), was passed through the cartridge using the optimized method to determine the retention time and recovery of each analyte in the MISPE. Very good separation of chromatographic peaks for the two analytes were obtained. SAL was eluted at 5.97 min. while clenbuterol was at 21.8 min. Recoveries showed that SAL elution (95 %) was not affected by clenbuterol (90 %). Thus the prepared MISPE can be useful for sample clean-up of both compounds.

3.3 HPLC method validation

Calibration curve of SAL using standard solutions ranging from 0.1 to 2.1 µg/mL using MISPE exhibited a good linearity with correlation coefficient (R) of

0.9965. The calculated LOD and LOQ of the method for HPLC were found to be 0.08 µg/g and 0.25 µg/g, respectively. Acceptable % recovery obtained was 93.6% and 90.2% for the organic chicken meat sample spiked with 0.3 and 0.6 µg/mL SAL, respectively.

3.4 Application to real samples

The applicability of the developed MISPE sample clean-up method to real chicken sample application, was tried. The chromatogram observed for the spiked chicken meat sample before MISPE clean-up (Fig. 2) showed large peak of SAL, however after using the MISPE, SAL peak decreased. This supported sample clean up after MISPE process, and elimination of interferences from the matrix. An overlap of the matrix to the SAL peak could result to a false positive SAL detection on the chicken meat sample.

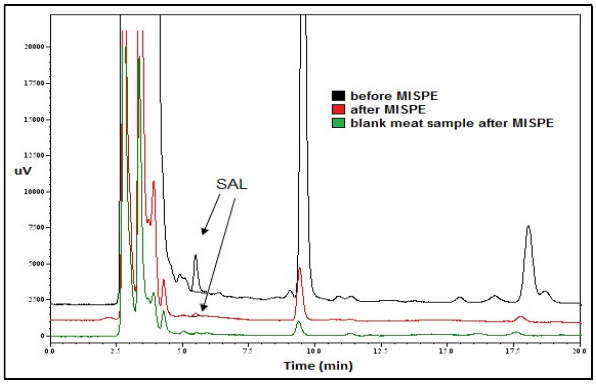

Figure 2. Comparison of chromatograms of spiked chicken meat sample before and after MISPE, and blank chicken meat sample

Three chicken meat samples including one from an organic chicken farm, one purchased from a wet public market and another from a local supermarket were analyzed for SAL. SAL was found below the detection limit for these samples.

4. CONCLUSION

This study was able to develop a molecularly imprinted polymer by precipitation polymerization that was capable of high binding affinity and selectively extracting capability for SAL in standard solution and real sample. The results showed that the developed MISPE is a potential competitive technique to the traditional SPE method in terms of selectivity and sensitivity.

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