

SAMPLE PREPARATION OF BETA-AGONISTS IN URINE, COMPARISON OF TWO DIFFERENT TECHNIQUES; MIXED-MODE SOLID PHASE EXTRACTION AND MOLECULAR IMPRINTED POLYMERS EXTRACTION

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Abstract

MIPs are engineered cross-linked polymers that can exhibit high affinity and selectivity towards specific compounds, e.g. beta-agonists. The interactions between MIP particles and analytes are stronger than interactions obtained in conventional SPE. Bond Elut Certify columns show a bi-model non-polar and strong cation exchange and are generally used for basic and neutral drugs in urine and other biological samples. Both types of clean up methods were optimized for more than 30 different beta-agonists. After optimization, both methods were fully validated according to Commission Decision 2002/657/EC.

Analysis is carried out by LC-MSMS, Ultima Triple Quad system.

Results indicate that both sample preparation methods can be used for a range of beta-agonists. However, none of these methods can be used for all compounds, e.g. nine compounds cannot be extracted with either method in the selected concentration range (1-5 $\mu\text{g L}^{-1}$).

Sample preparation using MIP columns gives generally cleaner extracts and shows thus less interference in chromatograms compared to Bond Elut Certify columns.

Introduction

Beta-agonists are a class of compounds which can be divided into groups with different chemical properties. Based on specific chemical groups beta-agonists can be divided into e.g. *hydroxyl-analin*, *anilin*, *phenol*, *hydroxyl-phenol* and other groups. This means it will be very difficult to develop a multi-component sample preparation method for all these compounds. Molecular Imprinted Polymers (MIPs) are designed to extract trace levels of analytes or analyte classes from complex biological matrices. In this study class selective MIP-SPE sorbent was used for multi-residue extraction of beta-agonists in urine samples and compared with a conventional SPE sorbent.

Bond Elut Certify is a bi-model non-polar and strong cation exchange SPE column, commonly used for basic and neutral compounds in urine, serum and other biological fluids. Due to its mixed-mode, effective sample clean-up and high recoveries can be obtained. In this study, a comparison is made between these two different approaches in sample preparation for beta-agonists in bovine urine. First, a LC-MSMS method was developed to analyze all compounds mentioned in Table 1. Secondly, the two sample preparations methods were optimized and finally the two methods were validated according to CD 2002/657/EC.

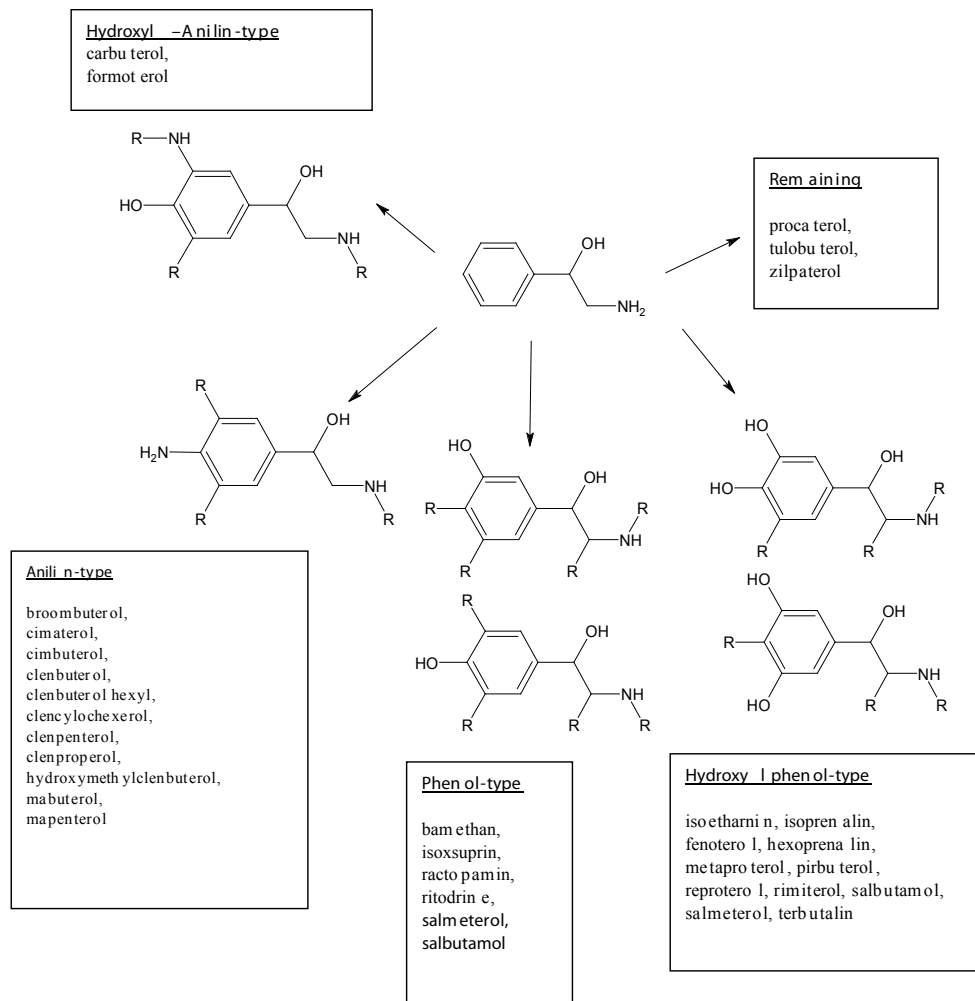


Figure 1: Common structures and classification of beta-agonists

Materials and Methods

The following columns were used in this study: SupelMIPS-beta-agonists, MIP-technologies, Sweden and Bond Elut Certify SPE columns, Varian Inc. All other chemicals were of analytical grade and were obtained from local distributors.

For this study blank bovine urine samples were spiked at several concentration levels (1-5 $\mu\text{g L}^{-1}$) with the beta-agonists mentioned in Table 1. Samples were hydrolysed using Helix Pomatia incubation, overnight at 37°C before sample clean-up. For quantification several deuterated internal standards were used. Details of the method are described in reference Baalvan et al. (2007).

Sample preparation MIPS

MIP columns are conditioned subsequently with 1 mL of methanol, 1 mL of water and 1 mL of 25 mM ammonium-acetate. The sample is loaded onto the column and washed with 1 mL of water, 1 mL of acetic acid/acetonitril (0.5/99.5 v/v) and 1 mL of 60% acetonitril. Compounds are eluted with two times 1 mL of acetic acid/methanol (10/90 v/v). The eluate is evaporated to dryness and redissolved in 100 μ l of methanol/water with 0.1% formic acid (5/95 v/v).

Sample preparation Bond Elut SPE

Certify columns are conditioned with 3 mL of methanol and 3 mL of 0.25M sodium acetate buffer pH 4.8. A sample is loaded onto the column and washed subsequently with 1 mL of 1M acetic acid, 6 mL of methanol and 2 mL of a acetone/chloroform mixture(1:1). Compounds are eluted with 7.5 mL of 3% ammonia (32%) in ethyl-acetate. The eluate is evaporated to dryness under a stream of nitrogen and redissolved in 100 μ l of eluents (5% methanol with 0.1% formic acid). The solution is placed in an ultrasonic bath for 5 minutes and centrifuged at 3000 rpm for 10 min.

LC-MSMS consisted of a Waters Alliance 2695, HPLC pump coupled to a Micromass Quattro Ultima triple quad. Molecular fragments of the compounds are determined by direct infusion technique. Compounds are separated on a Phenomenex, Luna C18, 3 μ m, 150x2.0mm I.D column. Eluent A: 5% methanol + 0.1% formic acid, eluent B: 95% methanol + 0.1% formic acid. Gradient starts at t=3 min and %B=0 -100 in 9 min.

Validation

After optimization of the LC-MSMS procedure, the methods have been validated according to CD 2002/657/EC, using an in-house validation protocol as described earlier by Kootstra et.al. (2007). Performance of the methods is expressed as CC α . Since LC-MSMS conditions are not changed, this parameter is directly related to the performance of the sample preparation technique.

Results and Discussion

The performance of the sample preparation methods, described as CC α , are presented in Table 1. This table also includes the reported CC α by the manufacturer of the MIP columns and the chemical class of the compound. None of the methods is capable to extract all compounds from urine samples. Both methods can be used for the analysis of several beta-agonists in urine samples. From the results as presented in Table 1, it is obvious that CC α values are lower for MIP-columns. Sample preparation using MIP-columns gives clean extracts without too much interference, especially when compared to SPE columns. From the chemical class of the compounds, it is concluded that MIPs are not suitable for most of the hydroxyl-phenol-class compounds. This confirms the three-dimensional structure of the polymers.

Table 1. Chemical class and CC α values obtained with MIP and SPE sample preparation.

Compound	Chemical Class	MIP CC α ng/g	Certify CC α ng/g	MIP CC α Manufacng/g
Bamethan	Ph	0.15	0.28	
Brombuterol	A	0.12	0.22	0.05
Carbuterol	HA	0.30	0.26	
Cimaterol	A	0.08	0.26	
Cimbuterol	A	0.06	0.23	
Clenbuterol	A	0.05	0.24	0.03
Clenbuterol-hexyl	A	0.06	0.22	
Clencyclohexerol	A	0.13	0.26	
Clenpenterol	A	0.11	0.20	
Clenproperol	A	0.04	0.34	
Fenoterol	HPh	0.35	0.22	
Formoterol	HA	0.15		0.07
<i>Hexoprenaline</i>	HPh			
Hydroxymethylclenbuterol	A	0.14	0.23	
Isoetharine	HPh			
Isoprenaline	HPh			
Isoxsuprine	Ph	0.13	0.29	0.10
Mabuterol	A	0.03	0.22	
Mapenterol	A	0.03	0.22	0.01
Metaproterol	HPh	1.85	0.94	
Pirbuterol	HPh			
Procaterol	O	0.30	0.22	
Ractopamine	Ph	0.08	0.25	0.03
Reproterol	HPh			
Rimiterol	HPh			
Ritodrine	Ph	0.13	0.25	
Salbutamol	Ph, HPh	0.19	0.24	
Salmeterol	Ph, HPh	0.15	0.25	0.19
Terbutaline	HPh	0.28	0.40	
Tulobuterol	O	0.08	0.25	0.05
Zilpaterol	O	0.23	0.27	

HA=hydroxyl-analin, A=anilin, Ph=phenol, HPh=hydroxyl-phenol, O=other

Hexoprenaline has no signal in the MS, so for further study hexoprenaline was removed from the method.

A difference between the two methods is the use of solvents for activation, rinsing an elution of the compounds; 8 mL for MIPs compared with 22.5 mL for SPE columns.

Another difference is that MIPs are eluted with an acid/ methanol as eluent while Bond Elut columns are eluted with a base/ ethylacetate as eluent. MIPs therefore make use of the neutral β -agonists form and Bond Elut columns the protonated form for binding.

The interaction between the MIP and the different compounds, might explain the difference in selectivity. As proposed by Widstand (2004), beta-agonists share a common structure as shown in Figure 1. The collective -OH and -NH group share a hydrogen bond with the acidic functional groups in the MIP. Therefore for the the aniline- and phenol-type β -agonists the MIP columns probably will work. The hydroxyl-phenol- group might not fit into the three-dimensional space or there is a competition between the common -OH groups and the

hydroxyl-phenol group for the same functional group The hydroxyl-phenol-group with it's polar structure will not fit into or bind more difficult to the reversed-phase material of the MIP. The more non-polar the β -agonists are the better they are bound to the column. This is probably due to the template or templates used for MIP production.

The data support the claim made by the manufacturer of the MIP Materials that there is a selective recognition mechanism for the analytes considered.

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