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Designs of experiments applied to salbutamol stability study in urine and different solutions in doping control

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Introduction: Salbutamol **(Fig. 1)** is a β_2-agonist commonly used for asthma treatment because of its bronchodilator effect. It is included in the prohibited list of the World Antidoping Agency (WADA) as a threshold substance, because of its therapeutic usage, with a permitted urinary concentration of 1000 ng/mL. This substance can be used in low concentrations for asthma treatment and in this case, it would not produce any technical advantage. This substance is unstable in some solutions as previously reported **[1,2,3,4].**

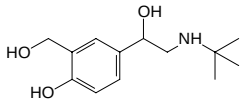

Figure 1: Structural formula of salbutamol.

This instability is critical in doping control analysis, because since salbutamol is to be quantified in urine samples, solutions used must have their exact concentration guaranteed. Designs of experiments (DOE) are normally used in order to understand the effect of desired factors with a minimum of experiments **[5].** Many factors might influence instability of a substance in solution and for this reason DOE were applied in this study. The main goals of this work were to determine whether salbutamol is unstable in other solvents as well as in urine. Also, the structural elucidation of the degradation products and the development of a better extraction method for salbutamol from urine were treated as objectives in the work since the screening procedure normally applied gives extraction yields of about 8 %. **Experimental:** Screening procedure for anabolic steroids and β_2-agonists consists of spiking a 2 mL aliquot of urine samples with internal standard (17α-methyltestosterone), enzymatic hydrolysis (β-glucuronidase from *E.coli*), followed by a liquid-liquid extraction with *tert*-butylmethylether (TBME) at pH 10 and derivatization with MSTFA:NH_4I:2-mercaptoethanol (1000:2:6/v:w:v). A 3 µL aliquot of this extract is analyzed by gas chromatography coupled to

mass spectrometry (GC-MS) using HP-1 (100 % poly-methylsiloxane) column, 0.25 mm ID; 0.11 μm of film thickness. In the development of a new extraction method, a complete two-level factorial planning was used to study three factors: the extraction pH, salt addition to the aqueous phase and the extraction solvent. A complete three-level factorial planning was then applied for the evaluation of the response surface of the method to the two most relevant factors. Extraction pH values evaluated were 8, 10 and 12 with carbonate buffer and potassium hydroxide; 2g of NaCl were added to all samples and the extraction solvent evaluated consisted of different proportions of an ethyl acetate : TBME mixture. Stability studies were performed in aqueous solutions (urine) and organic solvents (methanol and acetone). These studies were carried out by using design of experiments once more. Urine samples were studied at pH 3 and 11 and temperatures of -18°C and 40°C and analyzed by GC-MS at initial time, after 1 week and after 5 weeks. These temperatures were chosen because the use of DOE requires conditions so as to leave the region of interest in the middle of the model. Stability studies in organic solvents were performed with methanol and acetone. Samples were kept at temperatures of -18°C and 40°C and then analyzed by GC-MS at initial time, after one week and after 5 weeks. For structural elucidation of the degradation products chemical (CI) and electron ionization (EI) GC-MS were used along with ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) mono and two-dimensional experiments. **Results and discussion:** The extraction method developed shows extraction yields reaching 71% **(Table 1).** The optimal extraction conditions for salbutamol were pH = 12; Extraction solvent mixture 5 mL of ethyl acetate : TBME (20:1) and addition of 2 g of NaCl to the aqueous phase. Although pure ethyl acetate leads to slightly better extraction yields, the chosen mixture gives slightly cleaner extracts, which is why it is used instead of the pure solvent.

Table 1: Extraction yields of salbutamol in different conditions

Sample	pH	Extraction Mixture (Ethyl Acetate: TBME)	Extraction yield %
1	8	10:1	5.5
2	10	10:1	44.7
3	12	10:1	67.7
4	8	20:1	3.8
5	10	20:1	47.1
6	12	20:1	68.6
7	8	1:0	4.8
8	10	1:0	48.2
9	12	1:0	71.1

Stability studies in urine samples indicated reduction in salbutamol concentration **(Table 2)** while at least 3 different degradation products were formed**.**

Table 2: Percent concentrations of salbutamol in urine samples after 1 and 5 weeks in relation to the initial time analysis.

Temperature (°C)	pH	% salbutamol (after 1 week)	% salbutamol (after 5 weeks)
-18	3	90.1	85.6
40	3	85.0	80.1
-18	11	95.6	96.0
40	11	81.1	74.2

There is a pH influence on the formation of the degradation products in urine as one of the products is preferentially formed in alkaline and the other two are favored in acid pH values. All products are favored by elevated temperatures in the time studied, but occurred even at minus 18°C, however, at this temperature there was only the formation of the main product described below. Analyses of solutions, after 5 weeks storage under different temperatures, indicate a great reduction in salbutamol concentration (15% in the first week at 40°C) for the methanolic solutions and the formation of at least 3 degradation products different from the ones observed in urine samples. Acetone solutions, however, do not show the formation of any degradation products, which indicates that the possible mechanism for degradation requires a protic solvent (**Fig. 2**).

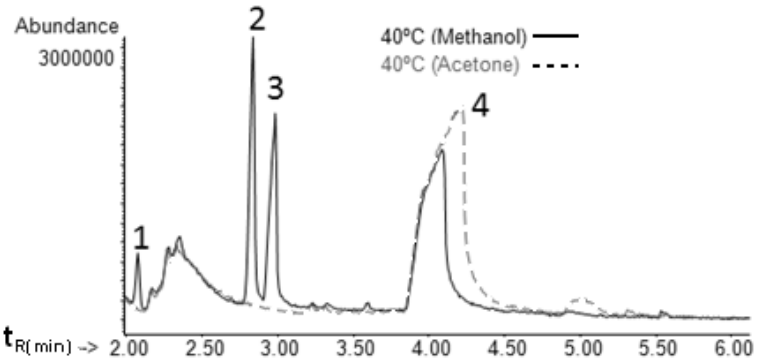

Figure 2: Overlaid ion chromatograms (*m/z* = 86) of salbutamol solutions in methanol and acetone. Degradation products (signals 1, 2 and 3) and salbutamol (signal 4) at 40 °C.

The proposed mechanism for the formation of the main degradation product in methanolic solutions is shown (**Fig. 3**). This mechanism explains why there are 3 degradation products as well as their elution order in GC. It is only possible to form the ethers by reaction of the two alcoholic hydroxy groups of the molecule, since the proposed mechanism would not be possible in the phenolic hydroxyl.

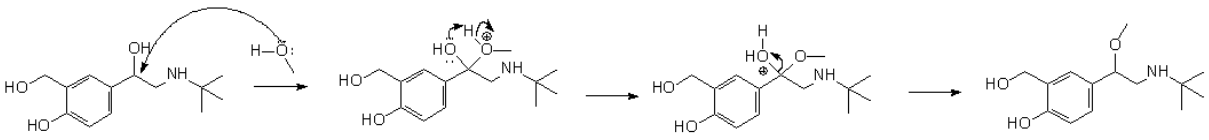

Figure 3: Proposed mechanism for the formation of the degradation product of salbutamol in a methanolic solution.

From **Fig. 2** it is noticeable that there are two degradation products (2.8 and 3.0 min) which elute earlier than salbutamol one near the other. This indicates that they are slightly less polar than salbutamol, having weaker intermolecular interactions and a consequent lower boiling point which leads them to elute earlier. These are probably the two *mono*-methyl products which had their molecular masses determined by GC-MS in CI mode. The main product was identified by NMR experiments as the ether represented on **fig. 3.** The third product **(Figure 2 – signal 1 – 2.0 min)** is probably the *bis*-methyl ether, which makes it even less polar, explaining why it elutes much earlier than the other two. **Conclusion:** Higher extraction yields were achieved with the method developed using DOE. A robust condition for extraction was also determined by DOE and the optimal extraction conditions were pH = 12; extraction mixture ethyl acetate : TBME (20:1) and addition of 2 g NaCl to the aqueous phase, achieving approximately 69 % of extraction yield. Stability studies in urine samples showed considerable reduction in salbutamol concentration after 5 weeks at both pH = 3 and pH = 11, especially when submitted to high temperatures. Also, degradation of salbutamol in methanolic solutions was detected with the formation of, at least, 3 different degradation products. Acetone was evaluated as an alternative solvent and gave satisfactory results, since no degradation was detected in its solutions. Based on the chromatographic behavior and molecular masses of the artifacts, the proposed substances are: 4-[2-(tert-Butylamino)-1-methoxyethyl]-2-(hydroxymethyl)phenol as the main degradation product, 4-[2-(tert-Butylamino)-1-hydroxyethyl]-2-(methoxymethyl)phenol and 4-[2-(tert-Butylamino)-1-methoxyethyl]-2-(methoxymethyl)phenol. A mechanism for the degradation in methanol was proposed. The present work shows that especial care must be taken into account regarding salbutamol quantitative analyses in urine samples, since degradation could lead to incorrect values, posing a great challenge to laboratories when trying to quantify this substance with accuracy.

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