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Detection of Salbutamol in Horse Urine

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1. INTRODUCTION

Salbutamol belongs to the group of $ß_2$-agonists. It shows their typical effects like relaxing smooth muscle. It is used as a bronchodilator in human and in veterinary medicine. The anabolic effect of salbutamol has been proved in humans (Martineau et al., 1992) and it is also likely in horses. In human sports salbutamol is allowed as an aerosol administration, whereas any other application is forbidden. In horse competition salbutamol is generally prohibited and considered as doping.

In this paper we present excretion studies which give information concerning the detection and the pharmacokinetics of salbutamol in horse urine. The analytical results may also be interesting for the detection of salbutamol in human urine.

2. EXPERIMENTAL

2.1 Excretion study

Three horses were treated with a single oral dose of salbutamol. The horses, two thoroughbreads (400 and 490 kg) and one halfbread (610 kg), were between four and five years old. Salbutamol was administered as Salbulair^R-tablets 2/4mg (3M Medica). A dose of 50 µg/kg was administered together with some food. Urine samples were collected for three days after application and a blank was collected before. The urine was collected after spontanous miction. 45 ml of urine were stored at 4°C and 45 ml at -20°C.

2.2. Experiments concerning the screening-procedure of salbutamol

Hydrolysis

In the initial experiments we used a pool of urine from the excretion study which was stored six months at 4°C.

Table 1

Kind of Hydrolysis	Concentration in urine [ng/ml]
Without hydrolysis	45
Acidic hydrolysis*	40
Hydrolysis with 50 µl Helix pomatia**	114
Hydrolysis with 50 µl ß-Glucuronidase from E.coli***	85

* For the acidic hydrolysis the pH is adjusted to 1 with 6N HCl, 10 mg of cysteine is added and the sample is incubated for 1 hour at 100°C.

** The pH is adjusted to 5.2 with 3M acetate buffer; Afterwards 50 µl arylsulfatase/ß-glucuronidase from Helix pomatia (Serva) are added and the sample is incubated for one hour at 50°C.

*** The pH is adjusted to 7.0 with 0.8 M sodium phosphate buffer. 50 µl of ß-glucuronidase from E.coli is added and hydrolysis is performed for 1 hour at 50°C.

The experiment was repeated with another urine from the excretion study which was frozen for six month at -20°C. The results were quite different from the pooled urine stored at 4°C.

Table 2

Kind of Hydrolysis	Concentration in urine [ng/ml]
Without hydrolysis	4
Hydrolysis with 50 µl Helix pomatia**	79
Hydrolysis with 50 µl ß-Glucuronidase from E.coli***	139

In the cold storage the concentration of free salbutamol in urine is significantly decreased. It is assumed that part of the salbutamol exists as an unstable conjugate which may be hydrolyzed during longterm storage. Furthermore, part of salbutamol is probably sulphate conjugated, because in the first experiment the recovery after hydrolysis with Helix pomatia was significantly higher than with ß-glucuronidase. After the second experiment it seems clear

that the activity of ß-glucuronidase in Helix pomatia is not sufficient to cleave all the glucuronides in horse urine. So its activity in horse urine has to be questioned. If the volume of enzyme is increased to 100 µl and the sample is incubated for 18 hours at 37°C, hydrolysis with Helix pomatia is more complete than with the same amount of ß-glucuronidase from E.coli. If the volume of enzyme is doubled again to 200 µl the recovery is even higher. To learn how to reach the optimal capacity with Helix pomatia enzyme one further experiment was performed. Two different amounts of Helix pomatia are added to the same urine and incubated for different time periods at 37°C. The yields are maximal when the urine is hydrolyzed for 24 hours with 100 µl Helix pomatia/5 ml urine.

Extraction

For the recovery experiments 5 ml of horse urine was spiked with 1µg of salbutamol. The extraction at pH 9.6 with tert.-butylmethyl ether showed a recovery of only 4%. An increase of the pH to 14 resulted in an even lower recovery. Also extraction with diethylether instead of tert.-butylmethyl ether did not show any advantage. On the other hand, the addition of tert.-butyl alcohol and sodium sulphate increased the recovery of salbutamol to nearly 100% (Table 3).

Table 3: Recovery of salbutamol with addition of sodium sulphate and/or tert.-butyl alcohol

Sodium sulphate [g]	tert.-butyl alcohol [ml]	Recovery [%]
-	-	4-5
-	1.0	30
4	-	35-40
4	0.5	50
4	1.0	90-100

Reextraction

In the screening procedure for basic compounds the organic layer is reextracted with 0.4 ml of 0.06N HCl, which serves as a very useful cleanup-step. To investigate reextraction, 1 µg of salbutamol is added to 5 ml of horse urine. After the reextraction the organic and the aqueous layer are evaporated, dried and derivatized seperately. This experiment shows if salbutamol goes completely into the aqueous layer. Indeed there was not any salbutamol in the organic layer. But the recovery in the aqueous layer was very low (about 20%).In later experiments it was shown that salbutamol is destroyed by addition of 0.06N HCl, which is also

the explanation for the low recoveries after acidic hydrolysis. So an acidic reextraction is not possible. As an alternative the reextraction was performed with 0.4 ml tartaric or citric acid (6 g/l). Again no salbutamol was found in the organic layer, but the recovery in the aqeous layer was even worse than with HCl (about 10%). As it appears that salbutamol is generally destroyed in an acidic environment, the reextraction is not utilized. The organic layer is transferred and evaporated to dryness under vacuum.

Drying in the vacuum desiccator

After evaporation the samples are dried in a vacuum desiccator over KOH/P_2O_5 .To check the stability of salbutamol under these conditions 500 ng was stored (dried) for different time periods in the desiccator. The recovered concentration decreased significantly with time.

Table 4: Recovery after different time periods in a vacuum desiccator

Time period [h]	Recovery [%]
2	91
5	90
21	81
48	52

A recovery of 100 % indicates the amount found after 10 minutes in the desiccator.

Derivatization

As salbutamol is easily TMS silylated at all three hydroxy goups, a catalyst is not necessary. The dry residue is derivatized with 100 µl MSTFA and heated for 25 minutes at 80°C. After this time salbutamol is completely derivatized yielding the tris-TMS compound. The secondary amine is sterically hindered and not silylated.

2.3 The salbutamol screening procedure

5 ml of horse urine is adjusted to pH 5.2 with 3M acetate buffer and 100 µl ß-glucuronidase /arylsulfatase (Helix pomatia) is added. The sample is incubated for 24 hours at 37°C. After hydrolysis, 50 ng of cloranolol (10 ng/ml urine) is added as an internal standard. The pH is than adjusted to 9.6 with 0.1 g KOH buffer (potassium carbonate/ sodium hydrogencarbonate 1:2,w:w). Extraction is performed with 6 ml of tert.-butylmethyl ether to which 1 ml of tert.-butyl alcohol and 4 g of sodium sulphate are added. The sample is shaken

for 20 min. and centrifuged. The organic layer is transfered to a silylated glass tube and evaporated to dryness under vacuum. Afterwards the sample is dried for one hour in a vacuum desiccator over KOH/P_2O_5. The dried residue is derivatized with 100µl MSTFA and heated for 25 minutes at 80°C.

2.4 GC/MS Parameters

GC/MS-system:	Hewlett Packard 5890 / Finnigan MAT 95
Carrier gas :	Helium (1 ml/min flow)
Split Ratio :	1:20
Septum Purge :	ca. 5ml/min
Interface :	Capillary direct
Injection Port :	capillary injection port
Analytical Column :	HP Ultra-1 fused silica capillary column, crosslinked methylsilicon (OV 1), 16m, 0.2 mm i.D., 0.11 µm film thickness
Injection Temperature :	300°C
Oven Temperature :	165°C
Temperature Program :	5°C/min till 195°C; 60°C/min till 320°C
Volume of injection :	1µl
Transfer Line :	300°C
Source Temperature :	240°C
Multiplier Voltage :	1.7 kV
Conversion Dynode	17 kV
Emission current:	1 mA
Electron energy :	65 eV
Dwell Time pro Ion :	148 msec
Cycle Time:	0.4 sec
Mass resolution :	3500
SCAN-Mode :	Electric

The samples are measured in HR-SIM using the masses m/z 86.0970 and 86.1096. Salbutamol tris-TMS and the internal standard cloranolol bis-TMS are registered with the fragment ion m/z 86.0970. This is the exact mass of the α-cleavage fragment $CH_2NHC(CH_3)_3$. The ion m/z 86.1096 is specific for $C_6H_{14}^{+\cdot}$ and is used to confirm the mass resolution.

3. RESULTS

3.1 Mass spectrum

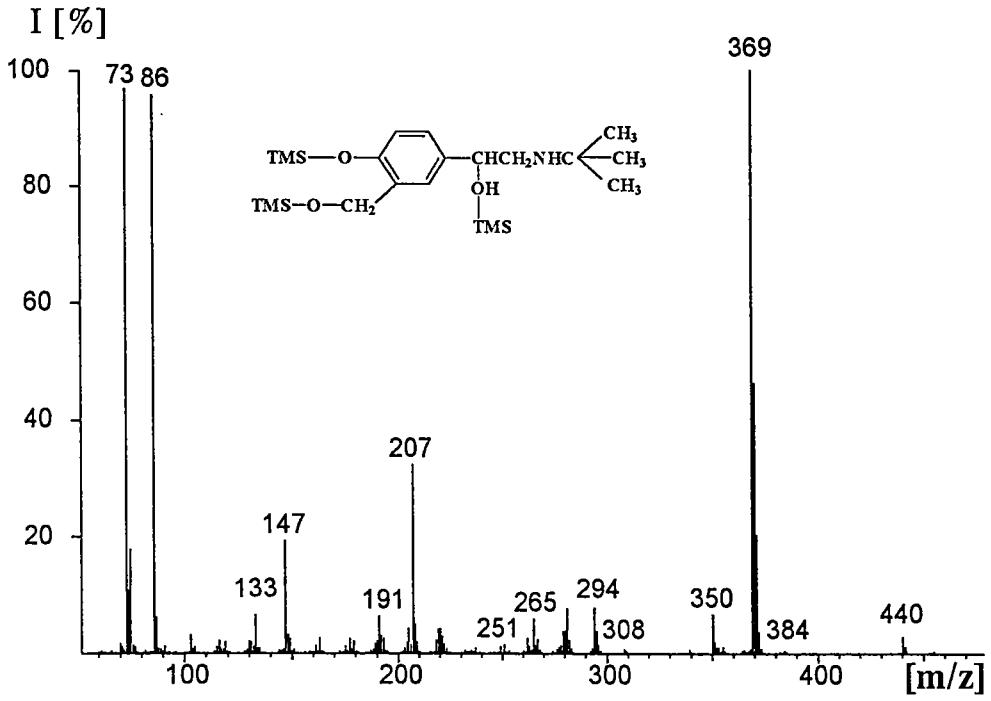

Fig.1: Structure and mass spectrum of salbutamol tris-TMS ($M^+ = 455$)

3.2 Concentration course in the urine

The concentration of salbutamol in urine increases rapidly after application. Already two hours after administration about 2 ng/ml were found in the urine. Three hours after the concentration increases to a value between 20 and 60 ng/ml urine. This means that salbutamol is rapidly absorbed and metabolized in the horse. The measured peak concentrations of 83, 155 and 126 ng/ml were reached between 8 and 24 hours after application. 60 hours after application there was still some salbutamol (about 2 ng/ml) in the urine which could be detected.

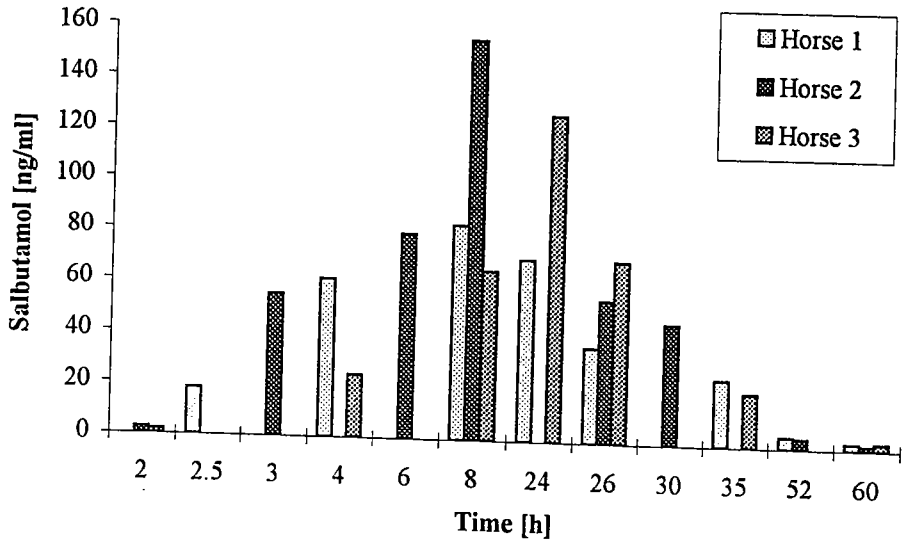

Fig 2: Urine concentrations of salbutamol after a single oral dose of 50 μg/kg

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