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Some Aspects of Investigation of Extraction, Derivatisation and Excretion of Terbutaline and Salbutamol
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Some aspects of investigation of extraction, derivatisation and excretion of Terbutaline and Salbutamol.

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INTRODUCTION

In human and veterinary medicine the β_2-agonistic drugs Salbutamol and Terbutaline are therapeutically used as bronchodilating agents. In addition to common action these substances are illegally used as growth promoters and anabolic agents. For this reason they are forbidden to use in sport with the exception of inhaling in the treatment of asthma or exercise-induced asthma. In the last years comprehensive scientific studies on β_2-agonists suggest various approaches to find adequate criteria for distinction between inhaled and oral use of these drugs [1-5]. The aim of this study is to investigate conditions for urine sample preparation and compare data obtained from analyzed urines after oral or inhaled application from healthy volunteers and asthma patients.

EXPERIMENTAL

A) STUDY OF THE EXTRACTION PROCEDURES

HPLC was used for measuring the extend of extraction at different pH-values, solvents and kind of extraction. The use of HPLC in this part of the studies allows chromatographic quantification without additional treatment of the samples for obtaining results regarding only the extent of extraction.

HPLC conditions – "Waters" liquid chromatograph, Column "NovaPack C18" 150x 39 mm, 5 μm, mobile phase 0.01M phosphate buffer (pH=3.5) and methanol (30:70 v/v) [6], flow 0.7 ml/min, λ=276 nm. The following calibration levels were used for quantification of Salbutamol and Terbutaline: 10, 50, 100, 150 and 200 ng/ml.

1. *Sample preparation for liquid/liquid (L/L) extractions* – The pH of 2 ml of urine spiked with a standard solution of Terbutaline or Salbutamol (200 ng/ml) was adjusted to the desired pH value with borate buffer standard solutions. 3 ml of organic solvent (diethyl ether, tert-butylmethylether (t-BME), t-BME + 0.5 ml t-BuOH, t-BME+ 0.5 ml t-BuOH+ 1 g Na_2SO_4, t-BME+ 0.5 ml i- PrOH) were added and the mixture was shaken for 20 min. The samples were centrifuged and the organic layer transferred to a fresh tube. The

organic solvents were evaporated to dryness under a stream of nitrogen at 40°C (water bath). The dried extracts were finally reconstituted in 100 µl HPLC mobile phase and 20 µl were injected into the HPLC system.

2. *Sample preparation for solid phase extraction (SPE)*

a) **XAD-2 column** – Standard column preparation, precondition and elution with 2 ml CH_3OH analogue to the screening procedure for anabolic steroids.

b) **C 18 column** – 3 ml Bakerbond C-18 column (J.T.Baker, Pilippsburg, NJ,USA), preconditioned with 2 ml H_2O and CH_3OH, extraction with 3 ml of methanol.

c) **Extrelute® column** – for 3 ml of sample solution (Merck, Darmstadt,Germany), preconditioned with 3 ml saturated solution of NaCl, elution with t-BME + 0.5 ml t-BuOH.

In all cases after SPE the organic solvent was evaporated to dryness. The dried extracts were finally reconstituted in 100 µl HPLC mobile phase and 20 µl were injected into the HPLC system.

B) DERIVATIZATION PROCEDURES

1) Silylation [7]: 100 µl MSTFA at 80°C, 10 min.
2) Selective derivatisation: 50 µl MSTFA at 80°C for 5 min followed by addition of 15 µl MBTFA and heated at 80°C for 10 min [8].
3) Methylation: with CH_3I/acetone/K_2CO_3 (30µl/150µl/10 mg) [9].
4) Cyclisation with methyl boronic acid: 0.25 mg methyl boronic acid were dissolved in 1 ml of ethyl acetate. 100 µl of this solution were added to the dry samples and kept at room temperature for one hour [10].
5) Silylation with N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA): 100 µl MBTSTFA at 80°C for 15 min [11].

C) *EXCRETION STUDIES*

1) Terbutaline: One volunteer with a single dose of 2.5 mg of Terbutaline sulphate ("Brikanil®", orally applied). The urine samples were collected up to 24 hours after application.

2) Salbutamol: The urine samples were collected according to the listed time schedule.

Time schedule for the collection of urine samples after administration of Salbutamol:

Sample	Health status	Administration	Time after administration
V1	Healthy volunteer	Orally "Salbutamol®" (Sopharma)	2 h after single dose 2.5 mg
V2	Healthy volunteer	Orally "Salbutamol®" (Sopharma)	3 h after second tablets of 2.5 mg
V3	Healthy volunteer	Orally "Salbutamol®" (Sopharma)	10 h after second tablets of 2.5 mg
H1	Hospitalized pat.	Orally "Ventolin" (Allen & Hanburys)	2 h after daily dose of 3x2 tabl. 7^{th} day
H2	Hospitalized pat.	Orally syrup "Ventoline"	2 h after daily dose of 4x5 ml syrup 6^{th} day
V4	Health volunteer	Inhaled "Ventoline" (Glaxo Welcome)	2 h after single dose of 0.2 mg (2 puffs)
V5	Health volunteer	Inhaled "Ventoline" (Glaxo Welcome)	5 h after single dose of 0.2 mg
V6	Health volunteer	Inhaled "Ventoline" (Glaxo Welcome)	7 h after single dose of 0.2 mg
H3	Hospitalized pat.	Inhaled "Ventoline" (Glaxo Welcome).	2 h after single dose of 0.2 mg
H4	Hospitalized pat.	Inhaled "Ventoline" (Glaxo Welcome)	2h after daily dose of 3x0.2 mg –7^{th} day
H5	Hospitalized pat.	Inhaled "Ventoline" (Glaxo Welcome)	2h after daily dose of 4x0.1 mg –4^{th} day

3) *Sample preparation for the excretion studies* – see Fig.1. Timolol was used as internal standard. For determination of the total amount of conjugated Salbutamol hydrolysis with Helix pomatia juice was used. Quantification of Terbutalineglucuronide was carried out as described in fig. 1 but hydrolysis with 25 µl glucuronidase from E. Coli at pH 7 (phosphate buffer) at 52°C for 1 h was used.

4) *GC/MS conditions*: HP 5890/HP 5970: Column – HP Ultra 1, 16 m, 0.25 mm i.d., 0.25 µm film thickness; Temperature program – 180 °C 20 °C/min – 310 °C, 4 min; Carrier: - 1 ml/min He; Split ratio 1:10; Transfer line – 280 °C; Injector – 280 °C. SIM mode for MTBSTFA derivatives (dwell time: 47 ms) – for Salbutamol – 495, 496,323, 86, 524; for Terbutaline – 482, 483, 322, 552,86; for ISTD– 86, 415, 373, 239,244.

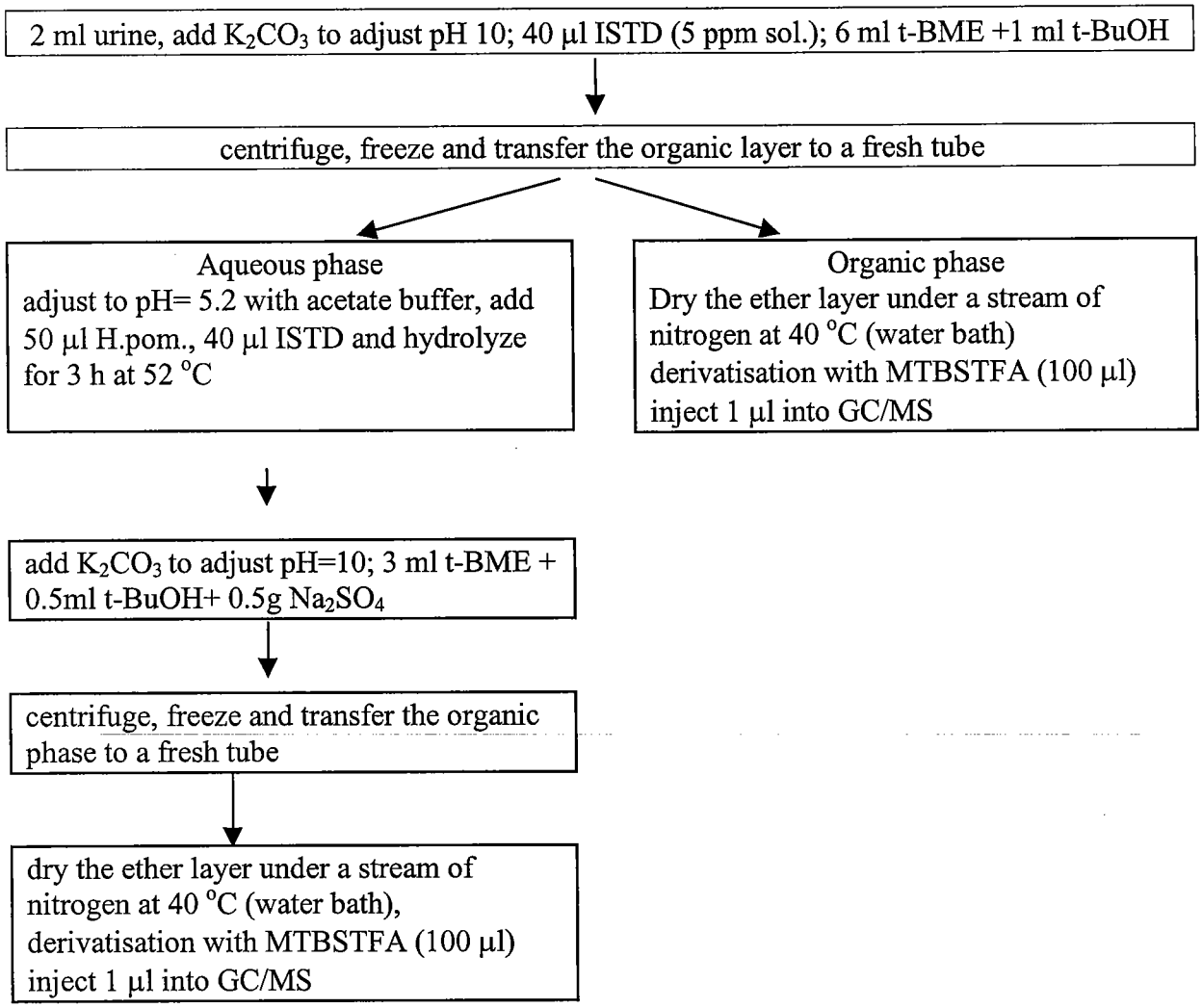


Fig.1: Sample preparation for quantitative determination of free and conjugated Salbutamol and Terbutaline in urine samples.

RESULTS AND DISCUSSION

Liquid/Liquid extraction (Fig.2): The results from the extraction experiments at different pH-values confirmed that in an alkaline range (pH 9-12) the extraction proceeds with better yield than in a neutral or acidic range (pH 3-4). The extractions performed with a mixture of t-BME+ t-BuOH and Na_2SO_4 gave higher yields of extraction than the other investigated solvents. The extraction recovery for Terbutaline was two to three times lower than for Salbutamol at the same pH-value.

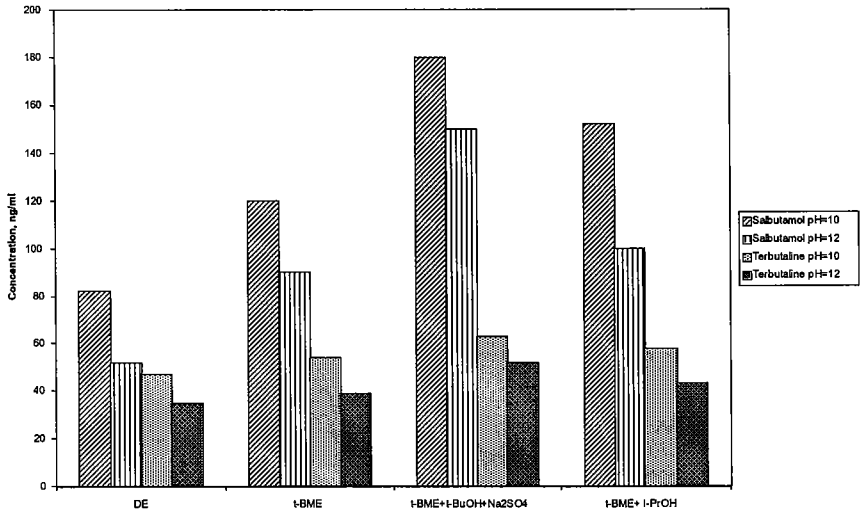


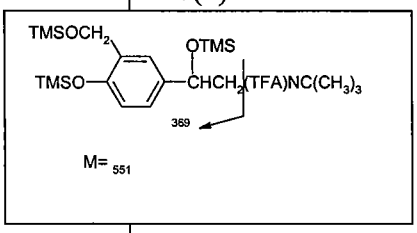
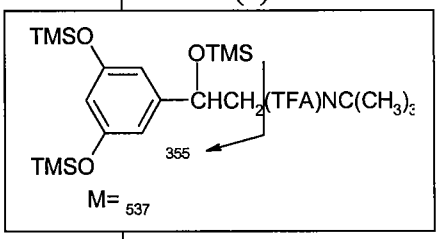
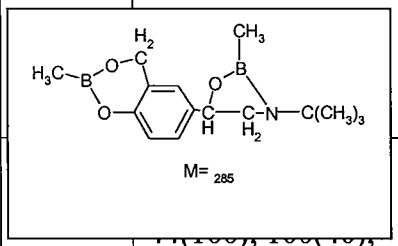
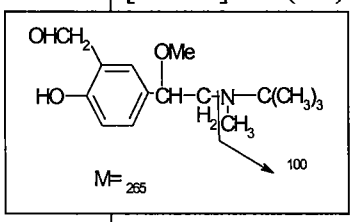
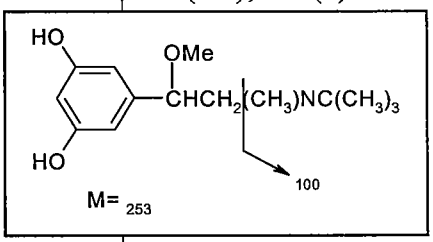
Fig.2. Liquid/Liquid extraction of Salbutamol and Terbutaline

Solid phase extraction (Fig.3)*:* The best recovery for Terbutaline (55%) was obtained using XAD-2 columns. SPE on C18 column was most efficient for Salbutamol among the tested columns. Comparing the data obtained from L/L and SPE extraction experiments L/L extraction with a mixture of t-BME, t-BuOH and Na_2SO_4 gave better recovery [4] than the studied SPE methods for Salbutamol. A better extraction recovery was achieved by SPE on XAD-2 for Terbutaline.

Derivatisation: Characteristic ions, retention times, relative abundances and structures of obtained derivatives for both substances are listed in Table 2

Table 2

Agents	Salbutamol		Terbutaline	
	t_R, min	Ions (rel. abundance)	t_R, min	Ions (rel. abundance)
1. MSTFA	3.58	369(100); 86(49); 371(18); 207(6); 404(4) M^+ 455(1)	2.35	356(100); 86(70); 426(9); 336(4); M^+441(0.02)
2. MTBSTFA	4.26	495(100); 496(43); 497(17); 524(3); 323(2.5); 86(5)	3.83	482(100); 483(23); 86(12); 322(3); 552(1.6); 510(1.6)

Agents	Salbutamol		Terbutaline	
	t_R, min	Ions (rel. abundance)	t_R, min	Ions (rel. abundance)
3.MSTFA/MBTFA	7.05	369(100); 390(6); 207(6.5); 281(2) M^+ 536(2)	6.48	355(100); 376(4); 466(3); 281(2); 239(2) M^+ 522(1)
				
4. Methane boronic acid	7.63*	230(100); 272(80); 271(47); 135(13); 174(8) M^+ 285(3)	-	No derivatives in applied conditions
				
5. CH_3I		[illegible] [M^+-15] 252(1.6)	5.67*	Dimethylated– 44(100); 100(46); (M^+-15) 238(1.6); 219(5)
				
	6.15*	Trimehylated- – 58(100); 192(7) [M^+-15] 266(1)	5.82*	Trimethylated- – 44(100); 100(30) [M^+-15] 252(3.3)
	6.99*	Tetramethylated- – 44(100); 100(43); [M^+-15] 265(2) ; M^+ 295(2)	5.99*	Tetramethylated- – 44(100); 100(44); (M^+-15) 265(4) ; M^+ 281(2)

* - Temperature program: from 140°C with 20°C/min to 310°C

A good stability and specific ions at higher masses of tert-butylmethylsilylether derivatives allow the discrimination of interfering ions form urine background which increases the limit of detection [12].

Excretion study: The concentration of free and conjugated Salbutamol in the samples are presented in Fig.4. After inhalation the amount of Salbutamol is lower than after oral administration, especially this differentiation is significant for free Salbutamol. The ratio of free/conjugated Salbutamol after inhalation (Fig.5) is lower than 0.6, but for a sample with

low oral dose(V1) it is very close to this observed border ratio. The experiments also show that variances between the amounts of free and conjugated Salbutamol in samples from healthy volunteers and hospitalized patients are not detected after same kind of administration with exeption of sample H5. The concentration of conjugated Salbutamol in this sample is much higher than in other samples obtained after inhalation. This patient recieved Salbutamol by nebulization and a part of condensed aerosol was swallowed.

In Fig.6 a curve of excretion after oral administration of a single dose of Terbutaline is shown. The amount of free Terbutaline is higher than the conjugated and of the sulfate conjugate is higher than of the glucoronide. This corresponds with literature data [14].

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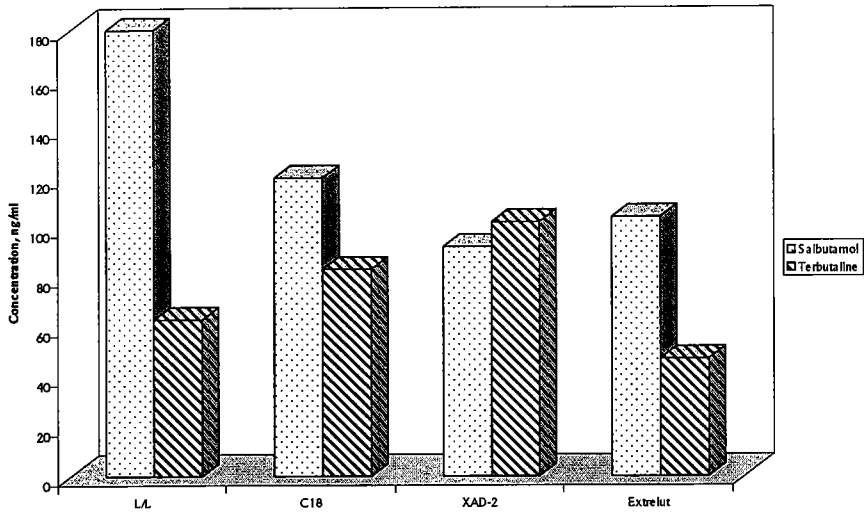

Fig.3. Quantity of extracted Salbutamol and Terbutaline from spiked urine (200 ng/ml) using different SPE columns at pH=10.

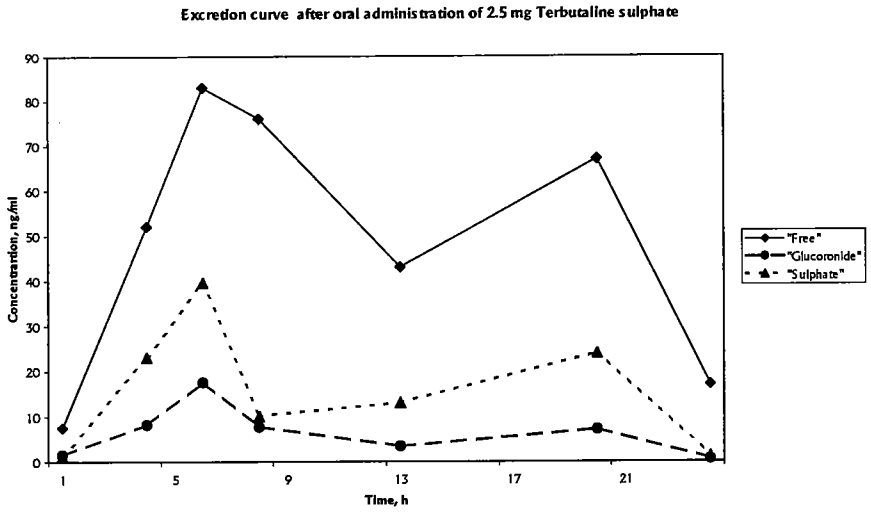

Fig.6

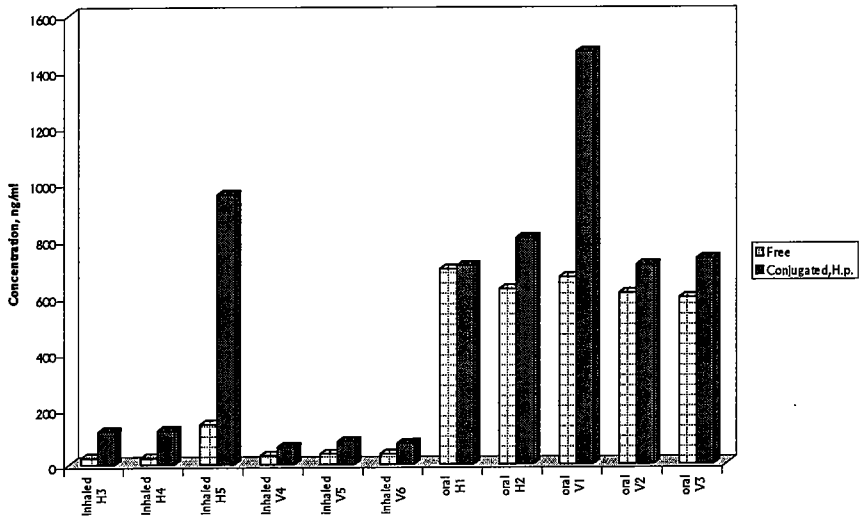

Fig.4. Concentration of free and conjugated Salbutamol in urine samples

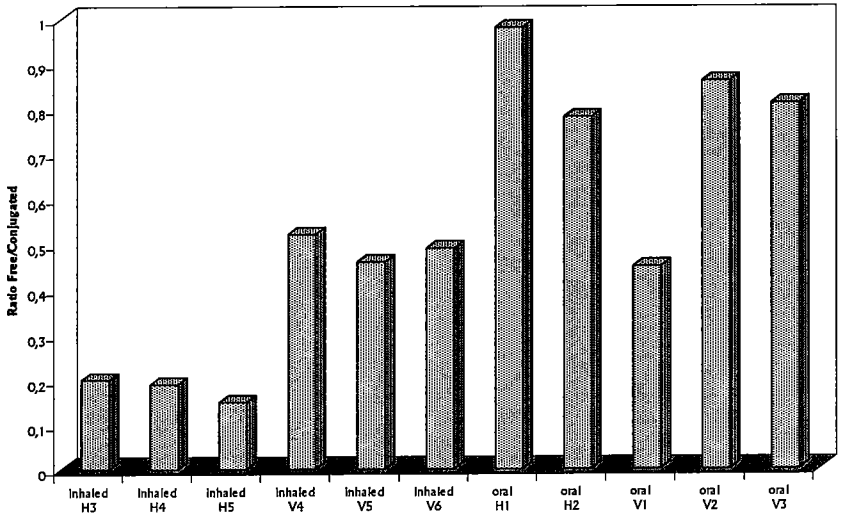

Fig.5. Ratio of free/conjugated Salbutamol in urine samples.