

Reprint from

RECENT ADVANCES
IN DOPING ANALYSIS
(8)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 2000

M.K.HENZE, G.OPFERMANN, H.SPAHN-LANGGUTH, W.SCHÄNZER:
Screening of Beta-2-Agonists and Confirmation of Fenoterol, Reproterol, Orciprenaline and
Terbutaline after Cyclisation with Formaldehyde
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in
doping analysis (8). Sport und Buch Strauß, Köln, (2000) 59-67

M.K. Henze^a, G. Opfermann^a, H. Spahn-Langguth^b, W. Schänzer^a

Screening of Beta-2-Agonists and Confirmation of Fenoterol, Reproterol, Orciprenaline and Terbutaline after Cyclisation with Formaldehyde

^a Institute of Biochemistry, German Sport University, Cologne, Germany

^b Institut für pharmazeutische Chemie, Martin-Luther-Universität Halle-Wittenberg, Halle (Saale), Germany

Introduction

Beta-2-sympathomimetic agonists are prohibited in sports due to their anabolic and stimulating side effects, but the administration of salbutamol, salmeterol and terbutaline are permitted by inhalation when prescribed for therapeutic purposes and when prior notification has been given to the relevant medical authority of the federation [1].

For fenoterol, orciprenaline, reproterol, ritodrine, salbutamol and terbutaline studies on biotransformation have been performed: fenoterol is excreted in human urine as its sulfate, conjugated with glucuronic acid and unconjugated [2, 3, 4, 5], orciprenaline unconjugated, as sulfoconjugate and as 2-isopropyl-4,6,8-trihydroxy-1,2,3,4-tetrahydroisoquinoline [4] and reproterol as free and conjugated 2-[3-theophyllinyl(7)-propyl]-4,6,8-trihydroxy-1,2,3,4-tetrahydroisoquinoline [6, 7, 8]. Ritodrine is transformed to its glucuronide and its sulfate. Additionally free ritodrine is excreted[9]. Salbutamol [2, 4] and terbutaline [2, 4, 10] pass unchanged and as sulfoconjugate. For the detection of beta-2-agonists in urine samples, substance specific methods are described [e.g. 11, 12, 13, 14].

The potential misuse as anabolic agent requires a method for screening analysis of many β -2-agonists in human urine.

As the tetrahydroisoquinoline derivatives are described as phase-I-metabolites, derivatisation with formaldehyde seems to be a suitable method for the detection of these compounds because the obtained derivatives are identical with the described metabolites. Only one product of each compound has to be monitored.

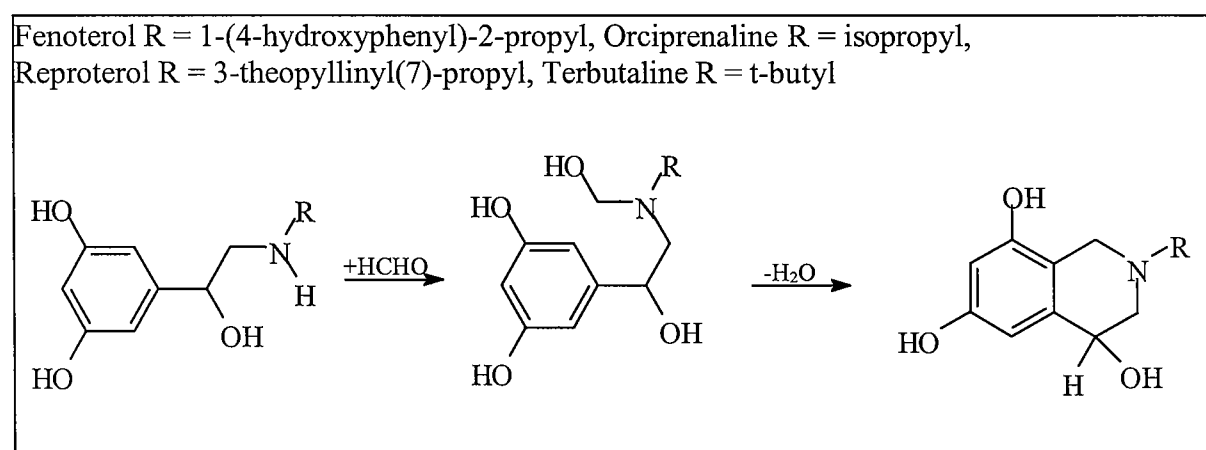
Derivatisation with Formaldehyde and Fragmentation Scheme of the Tetrahydroisoquinolines

The complete results are published in J. Chromatogr. B 751/1 (2001), 93-105.

Abstract:

A derivatisation step with formaldehyde converts fenoterol, orciprenaline, reproterol and terbutaline to one derivative, a tetrahydroisoquinoline via Pictet-Spengler reaction (figure 1), while other β -2-agonists remain unchanged.

Figure 1: reaction of the 3,5-dihydroxyphenyl substituted β -2-agonists with formaldehyde



Liquid-liquid extraction and trimethylsilylation follow (analogue to fig. 8). The tetrahydroisoquinoline derivatives show good gas chromatographical and mass spectrometrical behaviour. The other β -2-agonists are detected as parent compounds with the same recovery after sample preparation with and without formaldehyde. The EI mass spectra of the tetrahydroisoquinoline derivatives are shown (figure 2-5) and fragmentation schemes are proposed for the most intense fragment ions confirmed by measurements of spectra of deuterated derivatives (derivatisation with d_2 -formaldehyde instead of unlabelled formaldehyde) and daughter ion spectra.

Figure 2: mass spectrum of C,N-methylene-reproterol tetrakis-TMS,

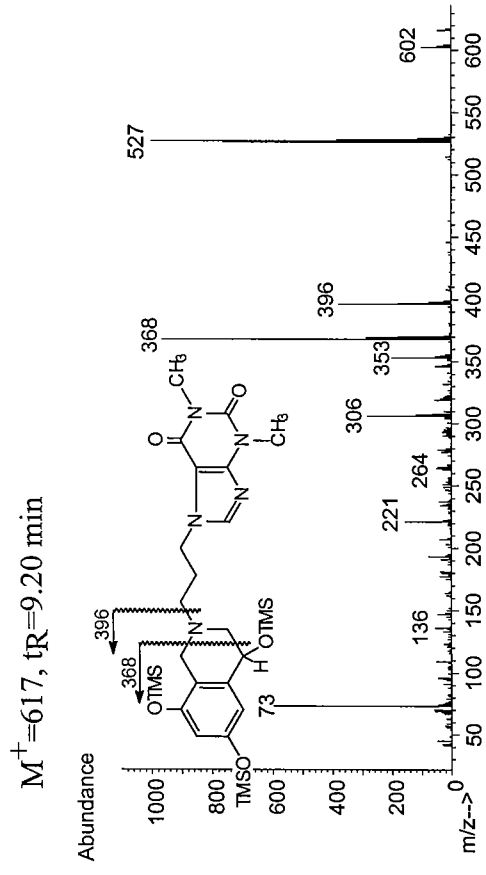


Figure 4: mass spectrum of C,N-methylene-oricprenaline tris-TMS,

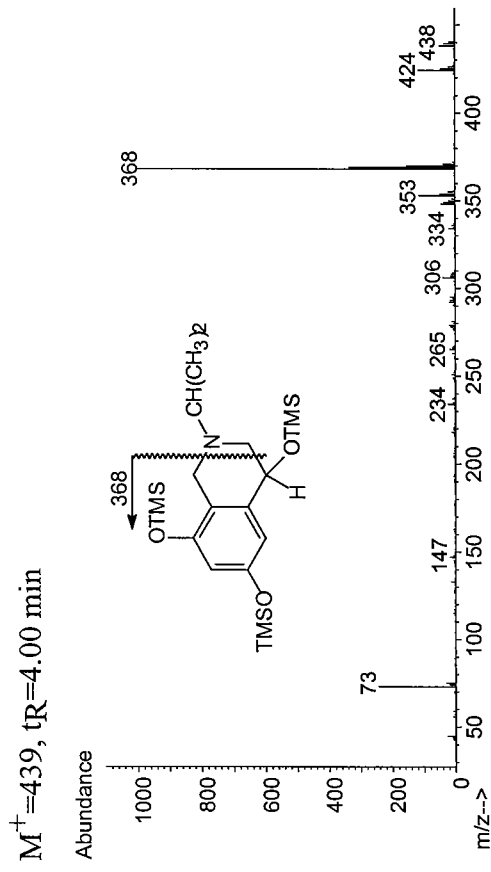


Figure 3: mass spectrum of C,N-methylene-fenoterol tetrakis-TMS,

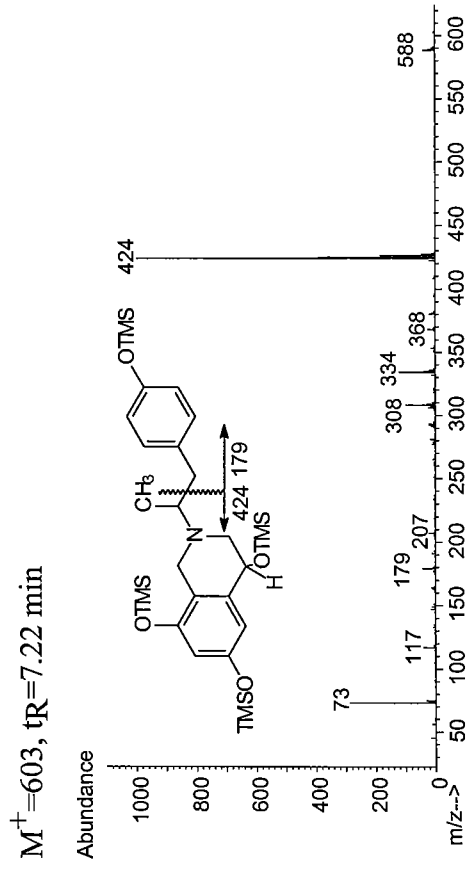
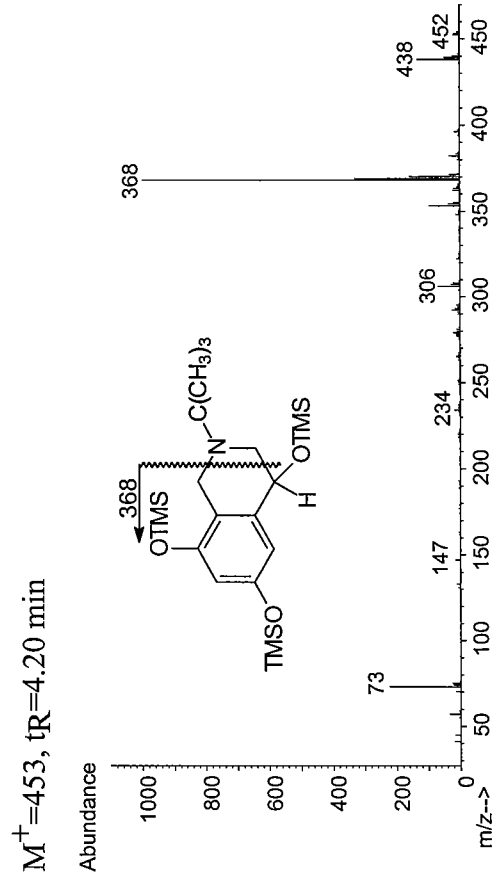


Figure 5: mass spectrum of C,N-methylene-terbutaline tris-TMS,



Comparison of Different Conditions for Hydrolysis

Experimental

Fenoterol hydrobromide (Berotec®, one tablet, 2.5 mg, subject A (male, 78 kg) and B (female, 67 kg)), orciprenaline hemisulfate (Alupent®, one tablet, 20 mg, subject A (male, 70 kg) and B (female, 58 kg)), reproterol (Bronchospasmin®, one tablet 20 mg, subject A (male, 70 kg) and B (female, 62 kg)), terbutaline hemisulfate (Contimit®, one tablet, 2.5 mg, subject A (female, 63 kg) and B (female, 61 kg)), salbutamol hemisulfate (Salbulair 2®, one tablet, 2.4 mg, subject A (male, 73 kg) and B (female, 61 kg)), and ritodrine hydrochloride (Pre-Par®, one tablet, 10 mg, subject A (male, 71 kg) and B (female, 62 kg)), were orally taken by volunteers.

5 ml of urine obtained from the excretion studies (0-8h urine) are analysed using different conditions for hydrolysis: a) no hydrolysis is included, b) after addition of 0.75 ml of phosphate buffer (0.8M, pH 7.0) and 50 µl of β-glucuronidase from *E. coli* hydrolysis at 37°C for 16 hours as well as at 50°C for 1 hour, c) after addition of 0.5 ml of Na-acetate buffer (4M, pH 5.2) and 50 µl of β-glucuronidase/arylsulfatase from *Helix pomatia* (HP-enzyme) hydrolysis at 37°C for 16 hours as well as at 50°C for 1 hour and d) after addition of 0.3 ml of hydrochloric acid (10 N) hydrolysis at 80°C for 1 hour are carried out.

After hydrolysis the pH is adjusted to 5.2 using acetate-buffer, a reaction of the mixture with 15 µl of formaldehyde (0.37% in H₂O) at 80°C for 3 h is carried out and the samples are extracted with a mixture of 5 ml of t-butyl methyl ether (TBME) and 1 ml of t-butanol (t-BuOH) after addition of NaCl (saturation) at pH 9.6. After centrifugation (750 g for 5 min) the organic layer is transferred and evaporated to dryness in vacuo.

100 µl of MSTFA/NH₄I/ethanethiol-TMS 1000:2:6 (v:w:v) are added and the sample is heated at 60°C for 15 min. The mixture is injected into the GC/MS system.

Instrumentation

Gas chromatography / mass spectrometry (GC/MS) analyses were performed on a Hewlett Packard (HP) 5890 gas chromatograph coupled to a Hewlett-Packard 5971 A mass selective detector (MSD) with the following parameters:

Injection parameters: volume: 3 µl, temp: 300°C

Column: HP Ultra1 (OV1): 16.8 m length, 0.2 mm I.D., 0.11 µm film thickness

Carrier gas: helium, split flow: 11 ml/min, head pressure: 0.62 bar, split 1:10

Oven temp.: 140°C with 20°C/min to 320°C final time 3 min

Ionisation: 70 eV electron impact ionisation (EI)

When the MSD was operated in the selected ion monitoring (SIM) mode the following ions were monitored: group 1: 1.40-4.43 min, m/z 438, 424, 369, 368, 356, 355, 335, 296, 291, 194, 100, 86 and 72, dwell time of 10 msec, group 2: 4.43-6.07 min, m/z 438, 407, 368, 346, 337, 335, 267, 241, 183, 178, 100 and 86, dwell time of 10 msec, group 3: 6.07-8.30, m/z 455, 424, 356, 355, 346, 322, 308, 277, 267, 250, 241, 236, 193 and 178, dwell time of 10 msec and group 4: 8.30-12.00 min, m/z 527, 369, 368, 356, 262, 250, dwell time of 24 msec. Scan mode: 40 – 650 amu, threshold 100, scan rate 2¹.

Results and discussion

The results obtained after analysis under different conditions of hydrolysis are shown in figure 6a-f. Best results for fenoterol are obtained after hydrolysis with the HP-enzyme, with acidic hydrolysis the obtained areas are a little bit smaller. For the other substances best results are obtained after acidic hydrolysis. Especially for salbutamol no gain is obtained after enzymatic hydrolysis. An additional clean up step by extraction with 5 ml of TBME at pH 1 prior to the reaction step with formaldehyde leads to cleaner extracts (figure 7) and improves the limit of detection. The resulting method for screening analysis of beta-2-agonists is shown in figure 8. The limits of detection are calculated using a signal-to-noise-ratio of 3:1. They are 0.4 ng/ml for salbutamol, 0.5 ng/ml for clenbuterol, 1 ng/ml for bambuterol and clenpenterol, 2 ng/ml for cimaterol, cimbuterol, fenoterol, mabuterol, orciprenaline, procaterol, reproterol, ritodrine, terbutaline and tulobuterol, 4 ng/ml for isoxsuprine, 8 ng/ml for salmeterol and 10 ng/ml for ractopamine.

Summary

A special sample preparation for beta-2-agonists is necessary to obtain suitable results. Only one derivative per compound needs to be monitored after reaction with formaldehyde. Best results are obtained by using acidic hydrolysis followed by an additional clean up by extraction at pH 0-1 and discarding the organic layer.

Figure 6a-d: comparison of different condition for hydrolysis of the phase II metabolites

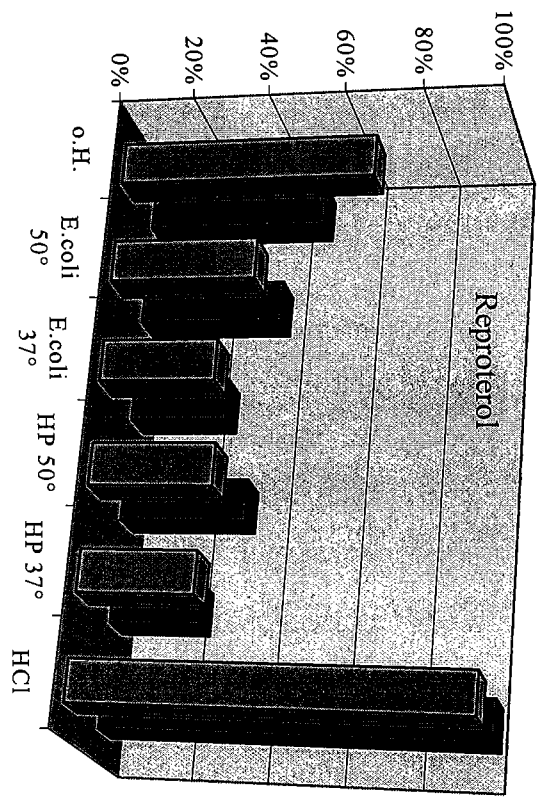
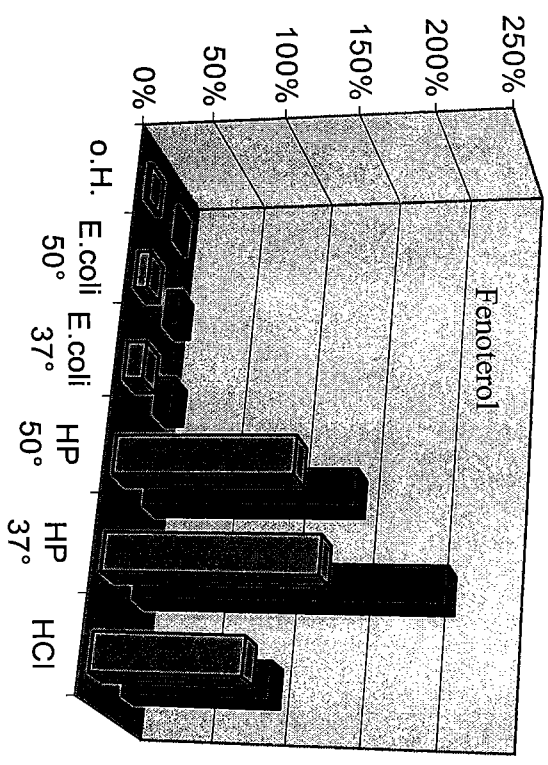
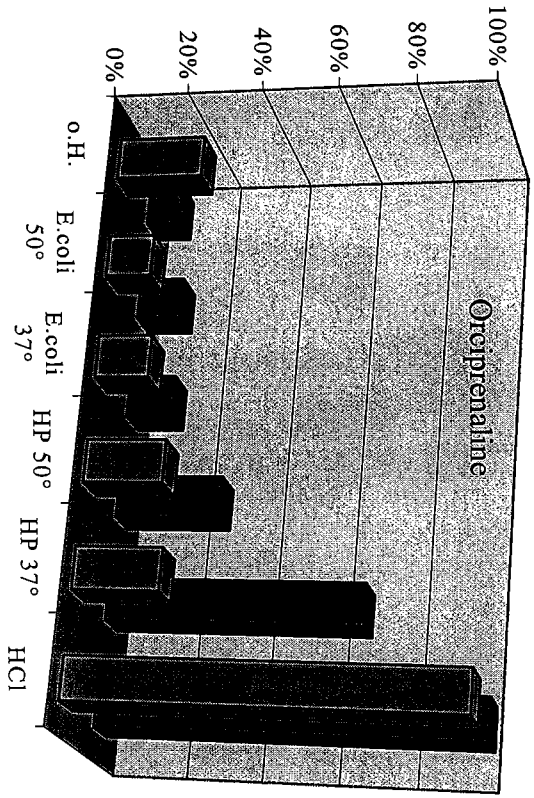
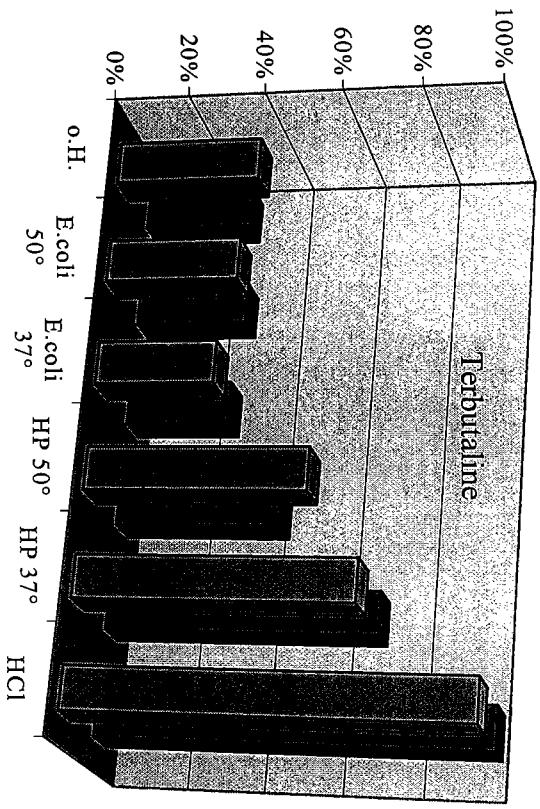


Figure 6e-f: comparison of different conditions for hydrolysis of the phase II metabolites

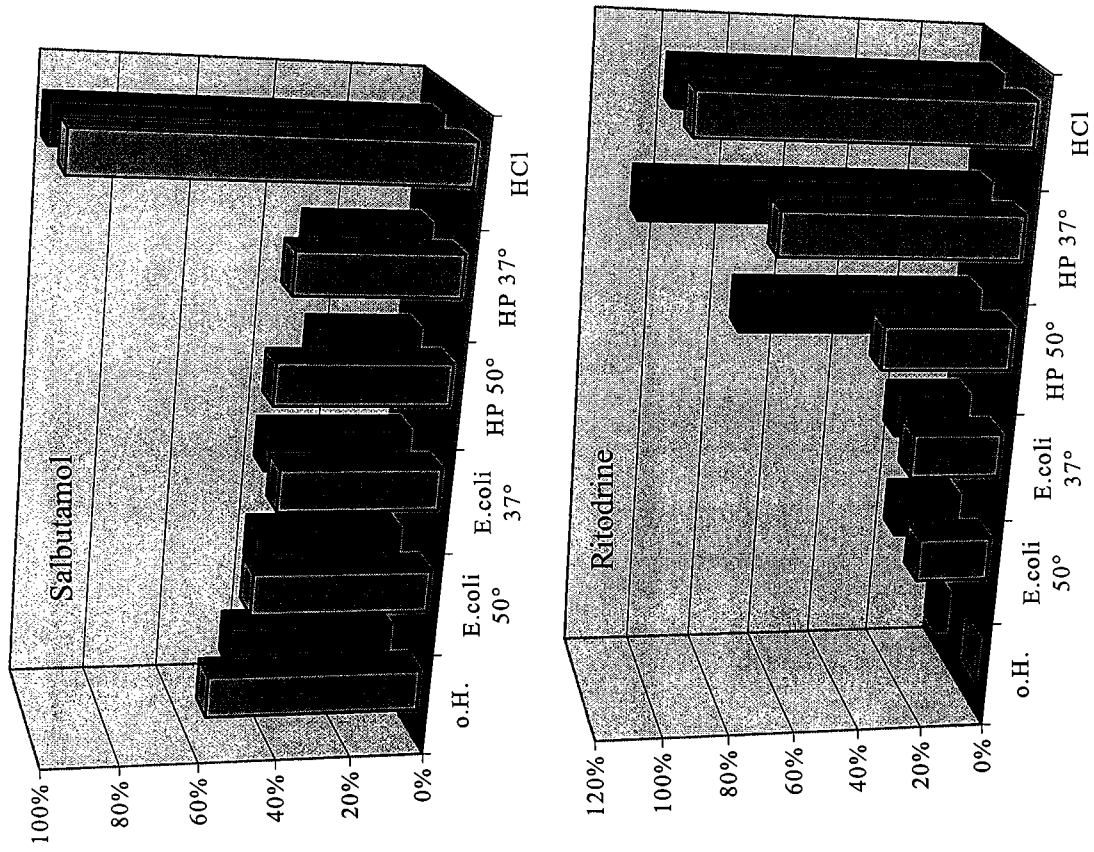
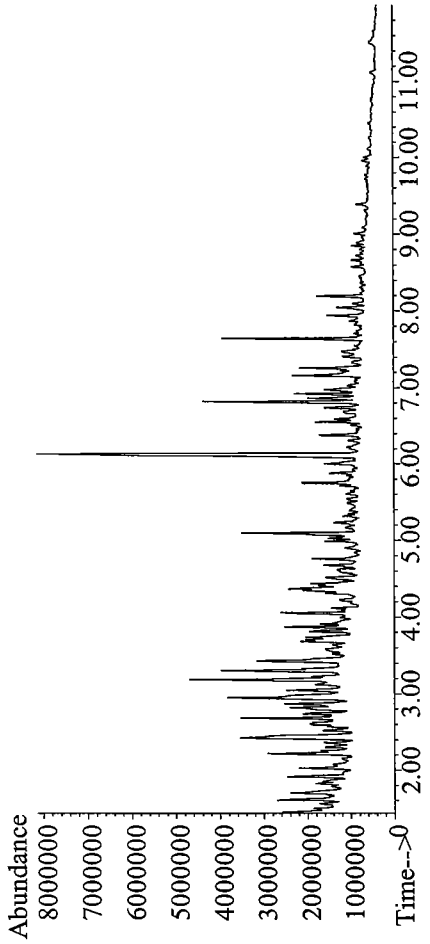


Figure 7: chromatograms (SCAN) of a fenoterol excretion study (0-8 hour urine)

after hydrolysis with HCl and preextraction with TBME at pH 1



after hydrolysis with HCl without preextraction with TBME at pH 1

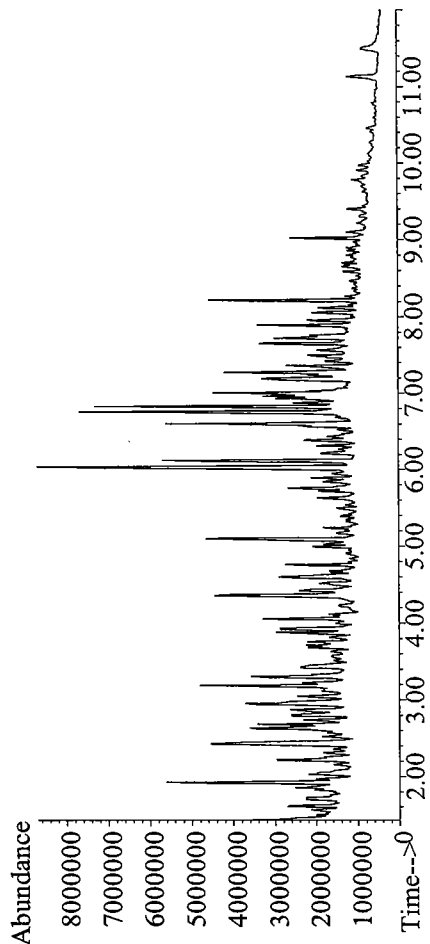
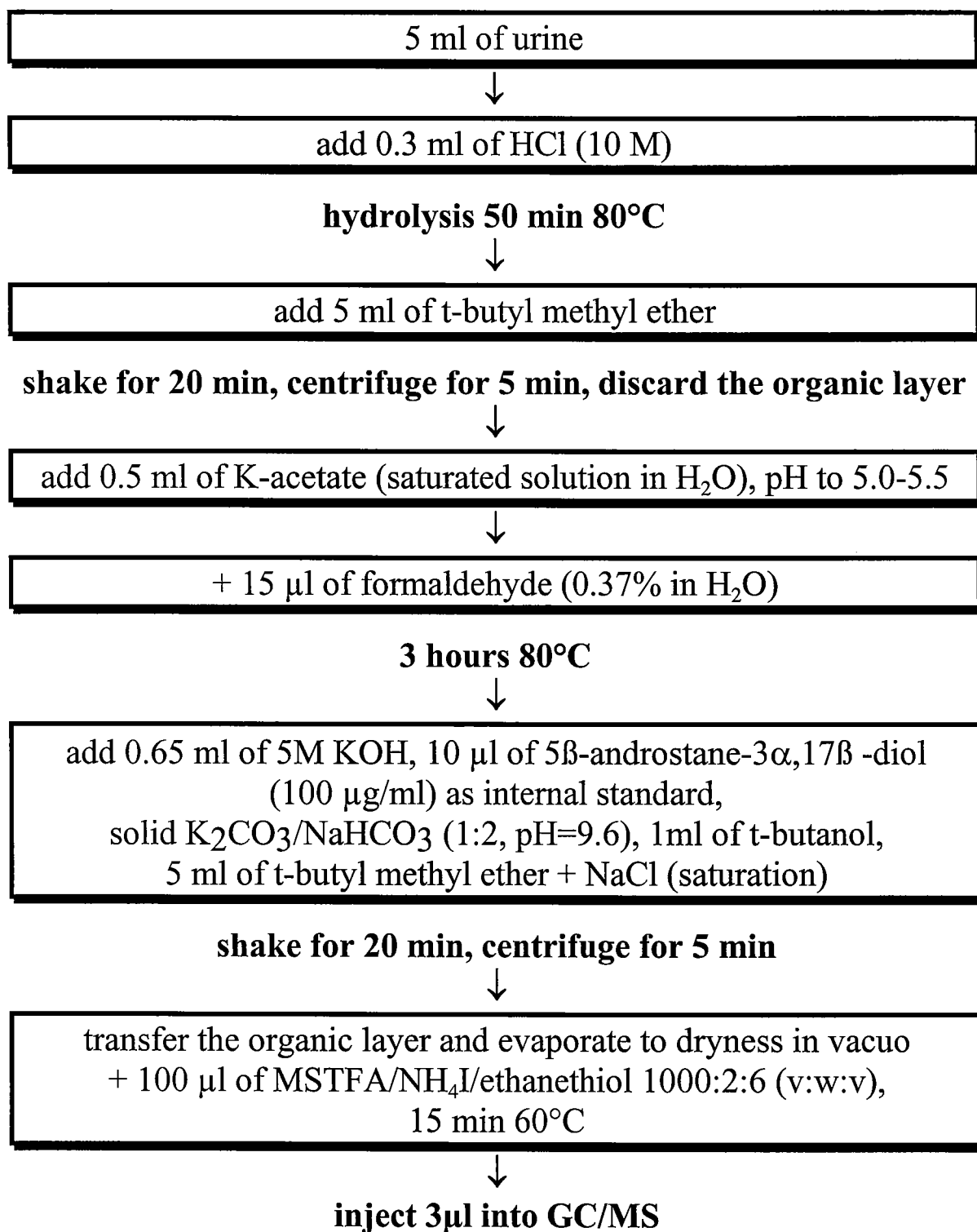


Figure 8: Sample preparation for screening analysis of beta-2-agonists



Acknowledgements

The Bundesinstitut für Sportwissenschaften, Köln, is acknowledged for financial support, ASTA Medica AWD (Frankfurt, Germany), Solvay Arzneimittel GmbH (Hannover, Germany), Abbott GmbH (Wiesbaden, Germany), Boehringer Ingelheim (Ingelheim, Germany) and Lindopharm GmbH (Hilden, Germany) for supplying the reference compounds. Thanks have also to be given to the volunteers of the excretion studies.

References

-
- [1] International Olympic Committee Medical Code, Prohibited Classes of Substances and Prohibited Methods, 1999
 - [2] G. Hochhaus, H. Möllmann, *Int. J. Clin Pharm Ther Tox* 30 (1992), 342
 - [3] R. Hildebrandt, B. Wagner, K. Preiss-Nowzohour, U. Gundert-Remy, *Xenobiot* 24 (1994), 71
 - [4] J. Bres, A.M. Clauzel, M.C. Pistre, H. Rachmat, F. Bressolle, *Bull Eur Physiopathol Respir* 21 (1985), 19s
 - [5] R.C. Heel, R.N. Brogden, T.M. Speight, G.S. Avery, *Drugs* 15 (1978), 3
 - [6] H. Sourgens, F.E. Köster, *Reproterol and Competitiv Sports - A Literature Review*, Focus Clinical Drug Development GmbH, Neuss, 1996
 - [7] G. Niebch, K. Obermeier, H. Vergin, K. Thiemer, *Arzneim. Forsch.* 27 (1977), 37
 - [8] G. Niebch, K.H. Klingler, G. Eikelmann, N. Kucharczyk, *Arzneim. Forsch.* 28 (1978), 765
 - [9] W.T. Brashear, B.R. Kuhnert, R. Wei, *Drug Metab. Dispos*, 18 (1990), 488-493
 - [10] K. Tegner, H.T. Nilsson, C.G.A. Persson, K. Persson, A. Ryrfeldt, A. *Eur J Resp Dis* 65 (Suppl 134) (1984), 93
 - [11] F.J. Couper, O.H. Drummer, *J. Chromatogr. B*, 685 (1996), 265
 - [12] H.J. Leis, H. Gleispach, V. Nitsche, E. Malle, *Biomed. Environ. Mass Spectrom.*, 19 (1990), 382
 - [13] C.G. Georgakopoulos, C. Tsitsimpikou, M.-H.E. Spyridaki, *J. Chromatogr. B*, 726 (1999), 141
 - [14] R.A. Clare, D.S. Davies, T.A. Baillie, *Biomed. Environ. Mass Spectrom.*, 6 (1979), 31