U.Mareck, G.Fußhöller, H.Geyer, N.Haenelt, M.Thevis, M.Kamber, R.Brenneisen and W.Schänzer

Temporal Indication of Marijuana Use

Institute of Biochemistry, German Sport University Cologne, Germany

Abstract

According to WADA rules the use of cannabinoids is forbidden in competition.
In doping control the detection of cannabinoid misuse is based upon detection of the pharmacologically inactive metabolite 11-nor-delta 9-carboxy-tetrahydrocannabinol-9-carboxylic acid (carboxy-THC) in urine. No accurate prediction of the application time is possible because carboxy-THC has a half-life of 6 days.
Consequently the adverse finding of carboxy-THC in doping control urine samples collected in competition may probably result from cannabis use out of competition.
Because of its shorter half-life the analysis of the pharmacologically active metabolite 11-hydroxy-delta 9-tetrahydrocannabinol (11-hydroxy-THC) may be of interest for the detection of cannabis misuse in competition.
Doping control urine samples from national and international federations which have been reported as adverse analytical findings for cannabis in 2005 and 2006 were additionally analysed by gas chromatography/mass spectrometry (GC/MS) for the presence of 11-hydroxy-THC. In 2006 three urine samples showed carboxy-THC concentrations greater than 100 ng/mL and additionally 11-hydroxy-THC levels higher than 3 ng/mL (suggested cut off level in literature for temporal indication of cannabis use). In 2005 twelve specimens showed carboxy-THC values higher than 100 ng/mL. Only two samples contained 11-hydroxy-THC at concentrations greater than 3 ng/mL. This result may depend on storage conditions and degradation of the analyte.
Controlled urinary cannabinoid excretion studies are needed to substantiate the suggested cut-off level of 3 ng/mL or to determine an adequate cut-off level for 11-hydroxy-THC in sports drug testing.

Introduction

The use of Cannabis is prohibited in sports competition. Carboxy-THC is presently the target analyte for the detection of cannabis misuse in doping control urine samples. The reporting threshold is fixed at 15 ng/ml (1).
In general a high number of adverse analytical findings for cannabis is reported by accredited laboratories: 11,7% in 2005 (2) and in 2006 up to June 10% by the Cologne laboratory. For the Swiss Anti-Doping commission the findings were more than 50% in 2005 (10 for cannabinoids out of 18 positive cases) (3).
Carboxy-THC is the major urinary metabolite which is pharmacologically inactive (4).
Its half-life time has been determined at approx. 6 days. This long period is due to its highly lipophilic nature (5).
It is well known that concentrations higher than 15 ng/ml for urinary carboxy-THC are possible for a long time period after a single application of cannabis (4).
Occasional users of cannabis had positive urine specimens for 3-4 days after receiving a standard dose of marijuana. Urine specimens of heavy marijuana users remained positive for cannabinoids for 7-10 days after last drug use. Therefore it is very difficult to predict when an individual was last exposed to marijuana or hashish based on the urinary excretion of carboxy-THC analysis by GC-MS in single urine specimens (4).
This means also that an adverse finding of an in-competition doping control urine sample may derive from cannabis use out of competition.
Because of its shorter half-life time the analysis of the pharmacologically active metabolite 11-hydroxy-THC may be of interest for the detection of cannabis misuse in competition.
Methods for the determination of carboxy-THC and 11-hydroxy-THC using liquid chromatography / tandem mass spectrometry or gas chromatography / mass spectrometry have been established (4-8). Most of them have been optimised for the analysis of human plasma (6-8), but for doping control purposes blood sampling is rather seldom, and the primary specimen collected for drug testing in sports is urine. Only few articles show analytical results for urine specimens from uncontrolled clinical settings (4,5). Hence, an assay enabling the temporal indication of cannabis (mis)use is required, preferably combined with existing screening procedures.

Experimental

Chemicals and reagents

All solvents and reagents were of analytical grade purity (Fluka, Buchs, Switzerland; Riedel de Haen, Seelze, Germany; Merck, Darmstadt, Germany).
tert-Butyl methyl ether (TBME) was purchased from KMF Laborchemie (St. Augustin, Germany) and distilled before use.
ß-Glucuronidase from *Escherichia coli (E. coli)* was supplied by Roche Diagnostics GmbH (Mannheim, Germany). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Macherey & Nagel (Düren, Germany).
11-nor-9-Carboxy-Δ^9-THC-D_9 (Promochem/Cerilliant, Wesel, Germany) and 11-Hydroxy-Δ^9-THC-D_3 (Promochem/Cerilliant, Wesel, Germany) were used as internal standards. As reference substances 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (Sigma-Aldrich, Steinheim, Germany) and 11-hydroxy-Δ^9-tetrahydrocannabinol (Sigma-Aldrich, Steinheim, Germany) were used. All solutions and buffers were prepared using deionized water (Water Lab System, Millipore, Eschborn, Germany).

Sample preparation

The sample preparation was modified from an assay for steroid analysis using GC-MS described by Donike *et al.* (9-11). Conjugated and unconjugated anabolic steroids were extracted from urine at pH 9.6 with TBME following enzymatic hydrolysis at pH 7.
Urine samples (2 mL) were fortified with the internal standards 11-nor-9-Carboxy-Δ^9-THC-D_9 (30 ng) and 11-Hydroxy-Δ^9-THC-D_3 (10 ng) each. The samples are buffered to pH 7.0 with 0.75 ml of a 0.8 M phosphate buffer (Na_2HPO_4 : NaH_2PO_4 ,1:2 ,w:w). Twenty-five microliters of *ß*-glucuronidase from *E. coli* were added. The mixture was incubated at 50°C for 1 h. After cooling to ambient temperature, a pH of 9.6 was adjusted by addition of 0.5 mL of an aqueous solution containing potassium carbonate and potassium hydrogen carbonate (20%, 1:1, w:w). 5 mL of TBME were added, the mixture shaken for 5 min and subsequently centrifuged at 1800 rpm for 5 min. The organic layer was transferred to a new glass tube, evaporated to dryness at 50°C using a rotary evaporator under reduced pressure, and the dry residue was derivatised with 100 µL of MSTFA/NH_4I/ethanethiol 1000:2:3 (v:w:v) for 20 min at 60°C.

Gas chromatography-mass spectrometry

Analyses were performed using a HP 6890 quadrupole mass spectrometer coupled to a HP 5973 gas chromatograph. A J&W Scientific Ultra I (OV-1) column was employed; length 17 m, I.D. 0.2 mm, film thickness 0.11 µm, helium carrier gas at a head pressure of 12 psi.

A 3-µL aliquot of the sample was injected into the GC system which was operated in the split (1:10) mode. The GC temperature was ramped as follows: initial temperature 200 °C, program rate 15°C/min to 310°C. The injection port and transfer line were heated to 300°C. The target analytes and deuterated analogues were registered between 3 and 5 minutes using the ions *m/z* 371.20 and 374.20 (20 ms dwell time) for 11-hydroxy-Δ^9-tetrahydrocannabinol / 11-hydroxy-Δ^9-tetrahydrocannabinol-D_3 and *m/z* 371.20 and 380.20 (20 ms dwell time) for 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid / 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid-D_9 .

Authentic urine samples

Doping control urine samples from national and international federations which have been reported as adverse findings for cannabis in 2005 and 2006 were analyzed by gas chromatography/mass spectrometry for the presence of 11-hydroxy-Δ^9-tetrahydrocannabinol. The respective urine samples have been stored at –20°C for 90 days after reporting and thereafter at 4°C.

Quantitative determination of

a) 11-Hydroxy-Δ^9-tetrahydrocannabinol (calibration curve)

A calibration curve was generated with the concentrations 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 ng of 11-hydroxy-Δ^9-tetrahydrocannabinol per mL of urine. As internal standard 11-hydroxy-Δ^9-tetrahydrocannabinol-D_3 (5 ng/mL) was used. Each calibration point was prepared and analyzed once. The peak area ratios of analyte and ISTD were utilized to calculate the correlation coefficient, intercept and slope. The source of the spiked urine specimens is a pooled blank urine obtained from 3 healthy male volunteers.

b) 11-Nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (one point calibration)

One urine containing 15 ng/mL 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (analyte) and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid-D_9 (internal standard) each was prepared three times and analyzed once. The peak area ratios of analyte and ISTD were used for the estimation of the carboxy-THC concentration, statistical evaluation and proof that the concentration of carboxy-THC in the respective urine sample is higher than the reporting threshold of 15 ng/mL. The expanded uncertainty is also considered for reporting.

Results and Discussion

In Figure 1 eleven doping control urine samples from 2006 with adverse analytical finding for cannabis are presented. This analytical result is consistent with the determination of a carboxy-THC concentration higher than 15 ng/mL. Figure 2 shows the 11-hydroxy-THC concentrations for the respective urine samples. In literature a cut-off concentration of 3 ng/mL for 11-hydroxy-THC is suggested (4). Three of the samples show carboxy-THC values higher than 100 ng/mL and additionally 11-hydroxy-THC values higher than 3 ng/mL. However, the ratio between carboxy-THC and 11-hydroxy-THC is no suitable appraisal criterion for temporal indication of cannabis application, because in urine of frequent users an accumulation of the long term metabolite carboxy-THC will occur.

In 2005 twenty-six analytical adverse findings for cannabis were reported. Twelve samples show carboxy-THC values higher than 100 ng/mL (Figure 3), whereas in addition only two of them show 11-hydroxy-THC values higher than 3 ng/mL (Figure 4). A possible explanation may be the fact that those urine samples were stored frozen for 90 days after reporting and thereafter kept at 4°C. Probably a time- and temperature-dependent degradation of 11-hydroxy-THC may have taken place.

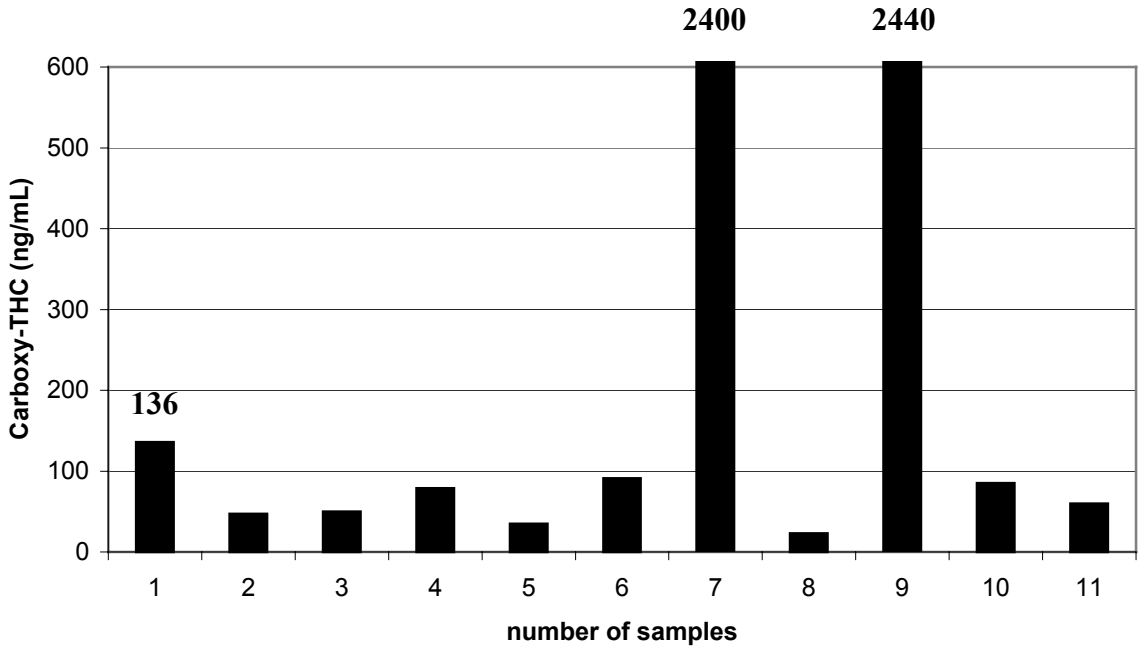

Figure 1: In competition doping control urine samples from 2006 with adverse analytical finding for cannabis (carboxy-THC > 15 ng/mL). Analytical results for carboxy-THC.

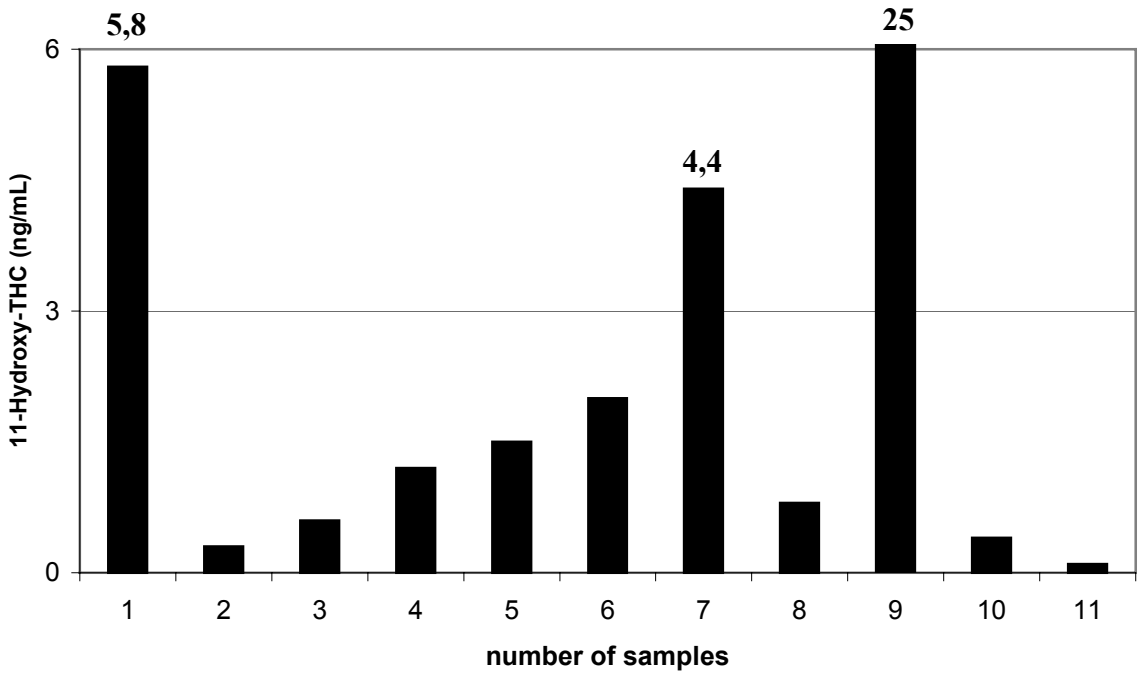

Figure 2: In competition doping control urine samples from 2006 with adverse analytical finding for cannabis (carboxy-THC > 15 ng/mL). Analytical results for 11-hydroxy-THC.

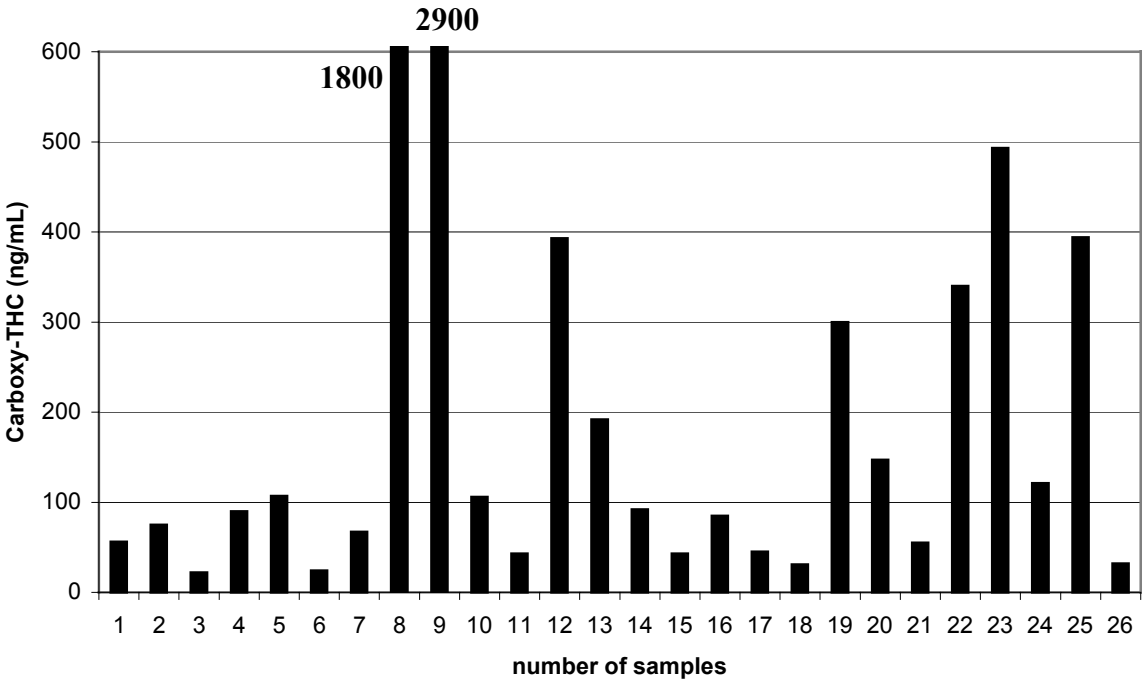

Figure 3: In competition doping control urine samples from 2005 with adverse analytical finding for cannabis (carboxy-THC > 15 ng/mL). Analytical results for carboxy-THC.

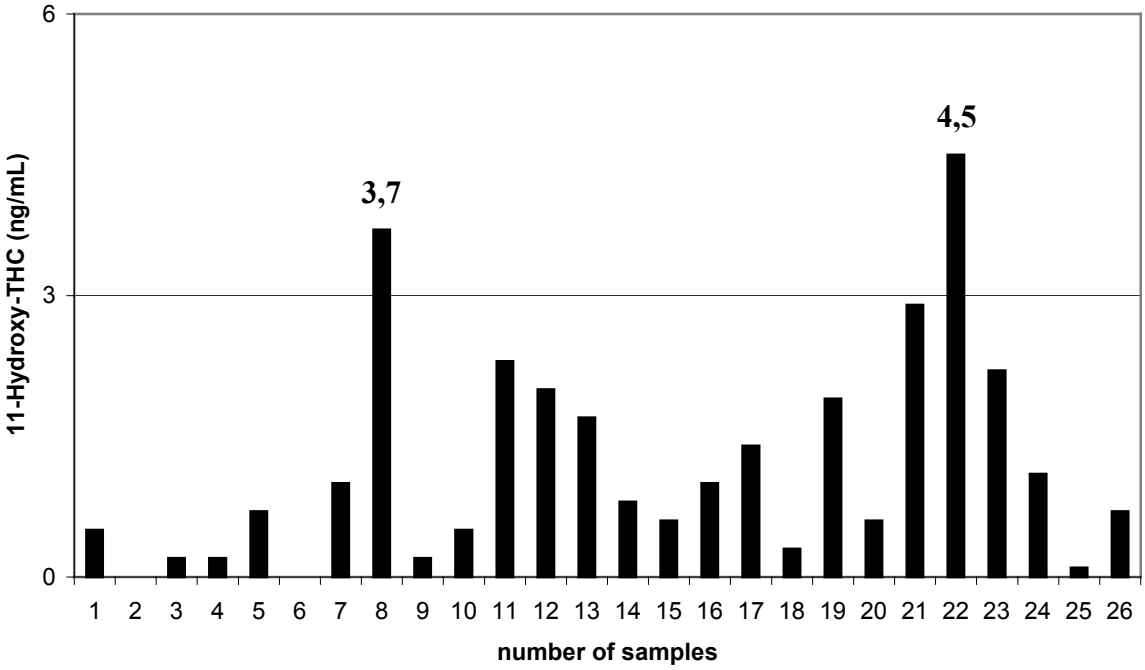

Figure 4: In competition doping control urine samples from 2005 with adverse analytical finding for cannabis (carboxy-THC > 15 ng/mL). Analytical results for 11-hydroxy-THC.

Summary/Conclusion

For the temporal indication of cannabis use the active cannabis metabolite 11-hydroxy-THC may be a suitable target analyte.
Further controlled urinary cannabinoid excretion studies are needed to confirm the suggested cut-off level of 3 ng/mL or to determine a convenient cut-off level for 11-hydroxy-THC.

Another suitable parameter for the temporal indication of cannabis use may be tetrahydrocannabinol (THC) itself. The psychoactive component of marijuana is excreted in urine as glucuronide conjugate. Urinary concentrations of THC greater than 1,5 ng/mL suggests marijuana use during the previous 8-h time period (5).
Analysis by means of LC-MS/MS may be the appropriate tool for the detection of THC-glucuronide in urine.

Acknowledgements
The study was carried out with the support of the Federal Office of Sports, Magglingen, Switzerland and the Manfred Donike Society e.V.

References

1. World Anti-Doping Agency: WADA Technical Document – TD2004 MRPL. Minimum Required Performance Limits for Detection of Prohibited Substances. Available: http://www.wada-ama.org/rtecontent/document/perf_Limits_2.pdf (online 05.07.06)
2. World Anti-Doping Agency: 2005 Adverse Analytical Findings Reported by Accredited Laboratories. Available: http://www.wada-ama.org/rtecontent/document/LABSTATS_2005.pdf (online 27.06.06)
3. Kamber M, Hintz O: Annual Report 2005, Anti-Doping Switzerland, p. 24.
4. Fraser AD, Worth D. Urinary excretion profiles of 11-nor-9-carboxy-delta 9-tetrahydrocannabinol and 11-hydroxy-delta 9-THC: cannabinoid metabolites to creatinine ratio study IV. *Forensic Sci Int.* 2004; **143**: 127.
5. Manno JE, Manno BR, Kemp PM, Alford DD, Abukhalaf IK, McWilliams ME, Hagaman FN, Fitzgerald MJ. Temporal indication of marijuana use can be estimated from plasma and urine concentrations of delta 9-tetrahydrocannabinol, 11-hydroxy-delta 9-tetrahydrocannabinol and 11-nor-delta 9-tetrahydrocannabinol-9-carboxylic acid. *J Anal Toxicol.* 2001; **25**: 538.
6. Maralikova B, Weinmann W. Simultaneous determination of Δ^9-tetrahydrocannabinol, 11-hydroxy-Δ^9-tetrahydrocannabinol and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol in human plasma by high-performance liquid chromatography/tandem mass spectrometry. *J. Mass Spectrom.* 2004; **39**: 526.
7. Gustafson RA, Moolchan ET, Barnes A, Levine B, Huestis MA. Validated method for the simultaneous determination of Delta 9-tetrahydrocannabinol (THC), 11-hydroxy-THC and 11-nor-9-carboxy-THC in human plasma using solid phase extraction and gas chromatography-mass spectrometry with positive chemical ionization. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2003; **798**: 145.
8. Nadulski T, Sporkert F, Schnelle M, Stadelmann AM, Rosner P, Schefter T, Pragst F. Simultaneous and sensitive analysis of THC, 11-OH-THC, THC-COOH, CBD and CBN by GC-MS in plasma after oral application of small doses of THC and cannabis extract. *J Anal Toxicol.* 2005; **29**: 782.
9. Donike M, Geyer H, Gotzmann A, Kraft M, Mandel F, Nolteernsting E, Opfermann G, Sigmund G, Schänzer W, Zimmermann J. Dope analysis. In *Official Proceedings of the International Athletic Foundation World Symposium on Doping in Sport*, Bellotti P, Benzi G, Ljungquist A (eds). IAAF: Florence, 1988; 53-87.
10. Geyer H, Schänzer W, Mareck-Engelke U, Nolternsting E, Opfermann G. Screening procedure for anabolic steroids – the control of the hydrolysis with deuterated androsterone glucuronide and studies with direct hydrolysis. In *Recent Advances in Doping Analysis*, vol.5, Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds). Sport und Buch Strauß: Cologne, 1998; 99-101.
11. Mareck-Engelke U, Geyer H, Schänzer W. Tetrahydrocannabinol (THC) in dope control. In *Recent Advances in Doping Analysis*, vol.7, Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds). Sport und Buch Strauß: Cologne, 1999; 51-60.