Reprint from

# RECENT ADVANCES IN DOPING ANALYSIS (12)

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Sport und Buch Strauß, Köln, 2004

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A. LEINONEN, K. VUORENSOLA, L.-M. LEPOLA, T. KUURANNE, T. KOTIAHO, R. KETOLA, R. KOSTIAINEN:
Applicability of Liquid-phase Microextraction (LPME) for Sample Preparation of Free Anabolic Steroids in Urine
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (12). Sport und Buch Strauß, Köln (2004) 329-333

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# Applicability of liquid-phase microextraction (LPME) for sample preparation of free anabolic steroids in urine

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## Introduction

Liquid-phase microextraction (LPME) is a relatively new sample preparation technique in which analytes in aqueous sample are first extracted to a thin layer of organic solvent in the pores of a hollow polypropylene fiber, and secondly to an acceptor phase inside the fiber [1,2]. The acceptor phase can be organic (two-phase system) or aqueous (three-phase system). Published LPME-applications cover mainly drug analysis and environmental monitoring [2]. In a comparison study of solid phase extraction (SPE), liquid-liquid extraction (LLE) and LPME of intact anabolic steroid glucuronides in urine, LPME showed the best selectivity and cleanest ion chromatograms in liquid chromatography–electrospray ionization tandem mass spectrometry (LC/ESI–MS/MS) [3]. This far, LPME has not been applied to sample preparation of unconjugated steroids.

In this study, the applicability of in-vial two-phase LPME was studied for the sample preparation of free anabolic steroids in urine. The extraction method was optimized with respect to the nature of organic solvent, extraction time, salting-out and temperature. The analysis of the samples was carried out either with LC/ESI–MS/MS or with gas chromatography–mass spectrometry (GC/MS). A novel LPME method with in-fiber

silylation, in which derivatization occurs during the extraction procedure without any other steps, was introduced in GC/MS analysis.

## Experimental

*Compounds*

Ethisterone (DNZm), 6β-hydroxy-4-chlorodehydromethyltestosterone (6CDM), 3'-hydroxy-stanozolol (3STZ) and 9α-fluoro-17α-methyl-androst-4-ene-3α,6β,11β,17β-tetrol (FLXm) were used as pilot compounds and methyltestosterone (MTS) as an internal standard.

*LPME*

Sample aliquot of 2 ml was transferred into a 4 ml glass vial and buffered with 0.4 ml of phosphate buffer (2 M, pH 7). After incubation with 20 µl of β-glucuronidase from E. Coli for 60 min at 50 °C, 0.5 ml of $K_2CO_3$/$KHCO_3$ solution (1:1, 20%) and 1.1 ml of purified water were added. A 6 cm piece of Accurel® polypropylene hollow fiber (600 µm id., 800µm od., pore size 0.2 µm, Membrana, Germany) was dipped in the acceptor phase (1-octanol, pentyl acetate or dihexylether) for 5 s and the excess solvent was removed by ultrasonication for 15 s. The fiber was filled with the acceptor phase and introduced into the sample solution. The sample was mixed with a magnetic stirrer at 1250 rpm during the extraction (15-90 min, 25-45 °C). For LC/MS analysis, the acceptor phase was collected and the fiber was flushed with 40 µl of the acceptor solvent. After evaporation to dryness, the residue was dissolved in the LC-eluent. For GC/MS analysis, a portion of the acceptor phase was directly injected into GC.

*Chromatography and mass spectrometry*

LC/ESI–MS/MS analysis was performed with a PE Micro LC and a Sciex API300 MS using earlier described operation conditions [4,5]. Injection volume was 5 µl and separation was carried out in a LiChroCART® Purospher RP C18e column (125 x 3 mm id., 5 µm). Eluent A contained 5 mM of ammonium acetate and 0.01% (v/v) of acetic acid in water, whereas eluent B contained the same additives in 90 % (v/v) methanol. A linear gradient was run with flow rate of 0.5 ml/min from 50% B to 100% B in 15 min and finally post-column split (1:100) prior to introduction to the MS source. Spray needle and orifice voltages were set to +5000 V and +20 V, respectively. Collision offset voltages were 15-60 V. The following precursor ion

→ product ion combinations were used for multiple reaction monitoring (MRM): m/z 372 → 95,337 for FLXm, m/z 351 → 147,209 for 6CDM, m/z 345 → 97,121 for 3STZ, m/z 313 → 97,109 for DNZm and 303 → 97,109 for MTS.

GC/MS analysis was carried out with an Agilent 6890/5973N using a HP-1 column (16 m, 0.2 mm id., 0.11 μm film). Injection of 2 μl was done in split mode (1:10) at 280 °C. Carrier gas was He (1 ml/min, constant flow). The oven was ramped first from 180 to 230 °C at 3 °C/min and then up to 310 °C at 30 °C/min and held at the final temperature for 3 minutes. MS was operated in selected ion monitoring (SIM) mode. Two specific ions for derivatized DNZm (m/z 456 and 301) and MTS (m/z 446 and 301) were monitored with dwell times of 50 msec.

**Results and discussion**

The efficiency of the different organic solvents in extraction of the free steroids from urine samples was tested both with traditional LLE and LPME (Table 1). The extraction recoveries (%) with LPME were lower than with LLE, which at least partly is due to the absorption of steroids in the polypropylene fiber. The recoveries of the more hydrophobic DNZm and 6CDM were better than with the more polar 3STZ and FLXm. The best extraction recoveries were obtained with 1-octanol, which was used for further optimization. For all compounds, 45 minutes was shown as the best extraction time. Either the increase in extraction temperature from 25°C to 35°C or 45°C or salting-out with 10, 20 or 30 % (w/v) NaCl did not affect yield. The most critical parameter in optimization was proper mixing of the sample solution during the extraction.

**Table 1**. Extraction recoveries (%) of the steroids in LPME utilizing different organic solvents.

| | 1-octanol (LLE) | 1-octanol (LPME) | pentyl acetate (LPME) | dihexylether (LPME) |
|---|---|---|---|---|
| FLXm | 67 | 2 | 0 | 0 |
| 6CDM | 110 | 36 | 7 | 1 |
| 3STZ | 89 | 5 | 0 | 0 |
| DNZm | 94 | 41 | 15 | 32 |

In LPME for LC/ESI-MS/MS analysis, extraction with 1-octanol for 45 minutes at 25°C was found as optimum. Figure 1 shows chromatograms obtained from the LC-MS/MS analysis of spiked urine. The analytes were detected well without any disturbance of the sample matrix. In LPME for GC/MS analysis, the fiber was pre-conditioned with dihexylether and then filled with MSTFA/$NH_4I$/dithioerythritol (1000:2:4, v/w/w), which was used as the acceptor phase. The extraction was performed at 45°C for 45 minutes. The extraction process led to silylation of the analytes. Ion profiles obtained from the GC/MS-SIM analysis of blank urine and urine spiked with DNZm are presented in Figure 2. The chromatograms were clean and no interfering peaks were observed.

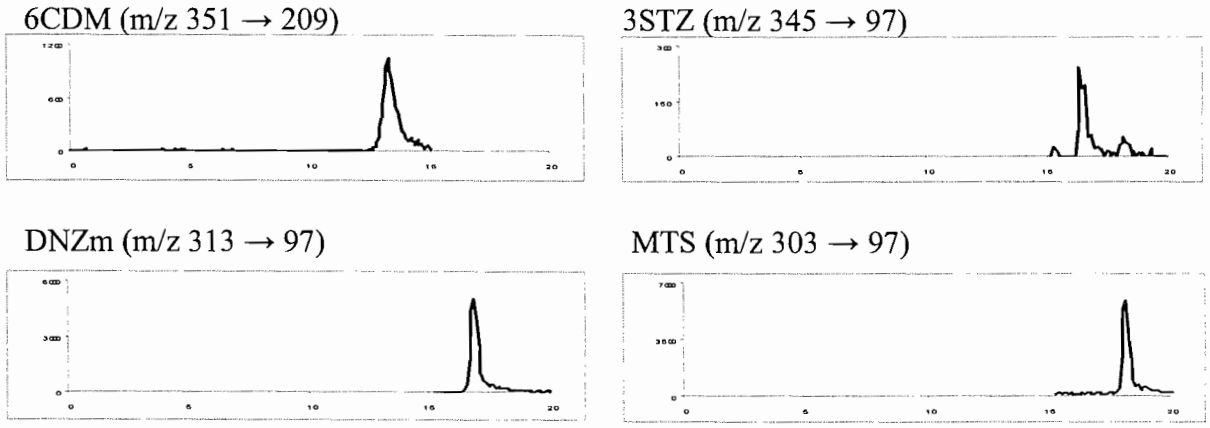


**Figure 1.** MRM chromatograms obtained from the LC/ESI–MS/MS analysis of urine spiked with 250 ng/ml of 6CDM, DNZm, 3STZ and MTS after 45 min LPME at 25°C into 1-octanol.

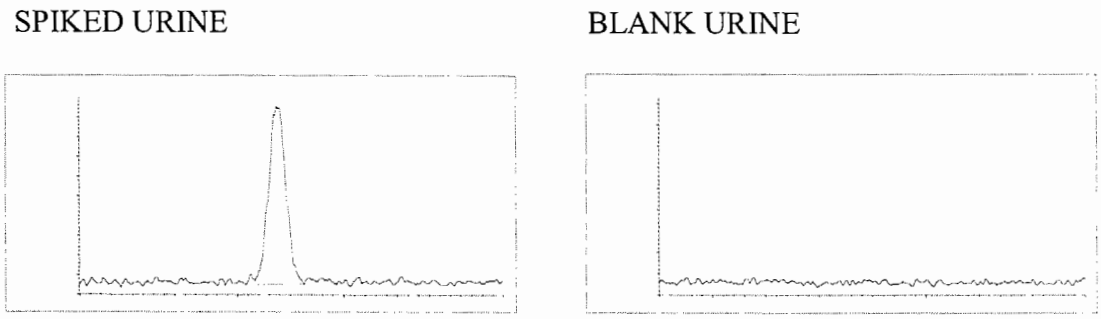


**Figure 2.** SIM chromatograms (m/z 456) obtained from the GC/MS-SIM analysis of blank urine and urine spiked with 20 ng/ml of DNZm after 45 min LPME at 45°C into MSTFA/$NH_4I$/dithioerythritol (1000:2:4, v/w/w).

## Conclusion

In the present work the applicability of two-phase LPME was studied for the sample preparation of free anabolic steroids in urine. As a conclusion, LPME can be combined both to LC/MS and GC/MS analysis. The selectivity of the extraction method is good, although, the extraction method is suitable only for the most hydrophobic steroids. LPME with in-fiber silylation for GC/MS analysis enables simultaneous filtration, extraction, clean-up, enrichment and derivatization of the hydrophobic steroids and would allow for greatly simplified sample preparation with lower cost.

## References

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