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Gas chromatographic/mass spectrometric determination of ethisterone in urine by liquid-phase microextraction with in-fiber silylation

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Introduction

In liquid-phase microextraction (LPME), analytes from aqueous sample are first extracted into a thin layer of organic solvent in the pores of a hollow fiber, and secondly into an acceptor phase inside the lumen of the fiber [1,2]. The acceptor phase can be organic (two-phase LPME) or aqueous (three-phase LPME). Published LPME-applications cover mainly drug analysis and environmental monitoring [2]. Recently the applicability of LPME has been studied for the sample preparation of both free and conjugated anabolic steroids in urine [3,4]. In general, fiber-based LPME provides good analyte preconcentration and excellent sample clean-up with relatively simple experimental set-up and low cost.

In this study, a novel sample pre-treatment method based on in-vial two-phase LPME with in-tube derivatization was introduced for gas chromatographic/mass spectrometric (GC/MS) analysis of ethisterone in urine. The method is based on previously published optimization studies [3] and it was validated regarding limit of detection (LOD), linearity, specificity, precision and accuracy.

Experimental

LPME

Urine 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 adding 20 µl of methyltestosterone (15 µg/ml) as internal standard, the mixture was incubated with 20 µl of β-glucuronidase (E. coli) at 50°C for 60 min. After enzymatic hydrolysis 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., 800 µm od., pore size 0.2 µm, Membrana, Germany) was preconditioned by dipping it in dihexylether for 5 s and the excess solvent was removed by ultrasonication for 15 s. The fiber was then filled with silylation reagent (MSTFA/NH_4I/dithioerythreitol, 1000:2:4 v/w/w) which was used as an acceptor phase, and introduced into the sample solution. The sample was mixed with a magnetic stirrer at 1250 rpm for 30 min at 45°C. A 2-µl portion of the acceptor phase was drawn up from the fiber and injected directly into GC/MS.

GC/MS

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 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. Ions m/z 301, 316, and 456 were monitored with dwell times of 50 msec.

Samples and method evaluation

In order to evaluate LOD, linearity and to calibrate the method, standards were prepared by spiking pooled human drug-free urine with ethisterone at concentrations of 10, 25, 50, 100 and 200 ng/ml. Standards were prepared and analyzed on 8 separate days. Calibration curves were obtained from the analyte to internal standard peak-height ratios using linear regression. To determine intra-day and inter-day precision as well as accuracy of the method, spiked urine samples at a concentration of 100 ng/ml were prepared and analyzed 12 and 8 times, respectively. Random interference of biological background was checked from drug-free urine samples of 4 male and 4 female volunteers.

Results and discussion

The method was linear between the calibration range 10-200 ng/ml. Calibration was repeatable with a good correlation coefficient of the curve (Table 1). Precision and accuracy of the method were calculated at concentration level 100 ng/ml. Intra-day and inter-day precision coefficient of variation (CV) was low and results were accurate (Table 2). LOD, with the criterion of a signal to noise ratio of 3, was estimated from ion profiles of m/z 456 being 1.8 ng/ml. Several aliquots of blank human urine were analyzed to check the selectivity of the assay. Based on the data obtained, chemical and biological background was repeatedly extremely low with no interfering peaks, which makes the interpretation of the data easy and reliable. Figure 1 shows chromatograms obtained from GC/MS-SIM analysis of blank and spiked urine samples. Ethisterone and methyltestosterone were detected as their bis-TMS derivates, indicating success of extraction to silylate the analytes.

Table 1. Statistics of 8 calibration curves. The method was calibrated on separate days with standards spiked in urine containing 10, 25, 50, 100 and 200 ng of ethisterone/ml.

	Slope	**Intercept**	**Correlation**
Mean	0.0216	0.0259	0.989
SD	0.0015	0.0441	0.0072
CV %	6.80		0.72

Table 2. Precision and accuracy of the method determined using urine samples spiked at 100 ng of ethisterone/ml.

	Intra-day	**Inter-day**
Added /ng/ml)	100.0	100.0
Mean (ng/ml)	96.2	101.0
CV (%)	2.7	7.9
Bias (%)	-3.8	1.0
n	12	8

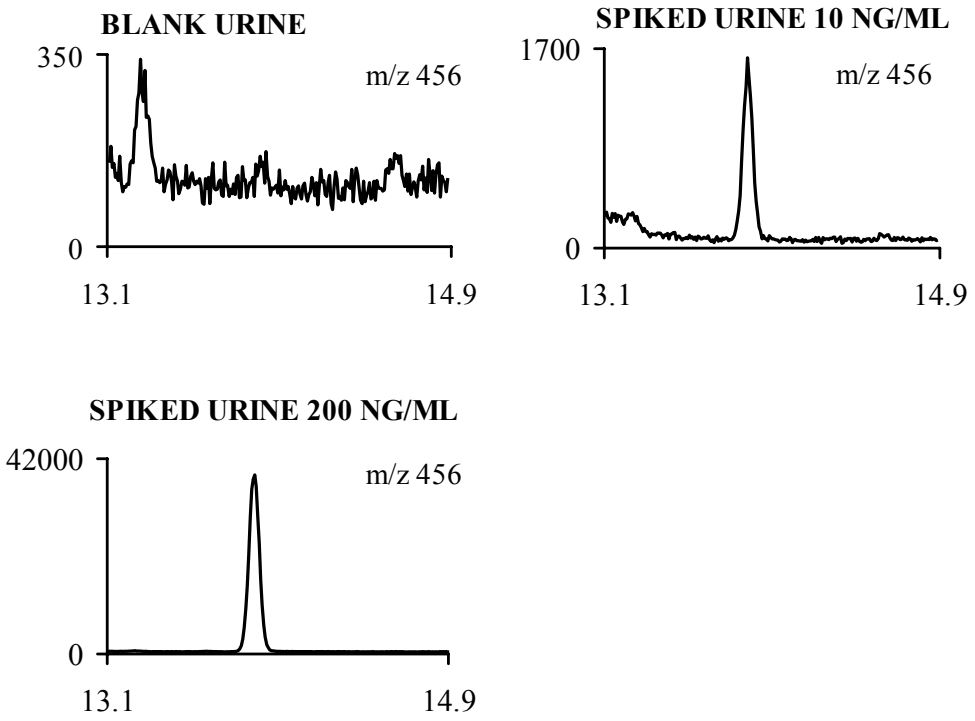

Figure 1. SIM chromatograms (m/z 456) of a blank urine, a urine spiked at 10 ng/ml and one spiked at 200 ng/ml (x-axis = time, y-axis = signal intensity).

Conclusion

The in-vial two-phase LPME with in-tube silylation allowed very simple but precise, sensitive and selective GC/MS analysis of ethisterone in urine. The method enabled simultaneous filtration, extraction, clean-up, enrichment and derivatization of the analyte without any other steps in sample pretreatment. The technique can dramatically simplify and enhance sample preparation of hydrophobic steroids and could be used for instance in pretreatment of samples in confirmatory analysis.

References

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