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Application of solid phase microextraction for the determination of stimulants, narcotics and other doping agents excreted free in urine

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INTRODUCTION

Solid-Phase Microextraction (SPME) is a relatively new technique that allows direct sampling of analytes from complex matrices. Its main advantages are simplicity, sensitivity and to be a solvent-free technique. It was firstly applied to environmental analysis, pharmaceutical products and in the food industry; successively it has been applied also to the determination of various drugs in different biological matrices both by submersion and head-space SPME [1-4]. So far, SPME was never applied to antidoping analysis, probably due to the huge number of samples to be analysed. The recent availability of dedicated autosamplers that allows automated sampling and subsequent injection in a GC/HPLC port renders this technique very attractive, especially whenever many samples per day have to be analysed.

This contribution proposes the use of SPME for the simoultaneous sampling, and subsequent GC/MS analysis, of most drugs/metabolites excreted free in the urine, i.e. stimulants, narcotics, local anaesthetics. Despite the change from IOC to WADA, stimulants and narcotics are indeed still banned; threshold substances are ephedrine and methylephedrine (prohibited at a concentration higher than 10 μg/mL) and cathine (>5 μg/mL), with caffeine and other ephedrines no longer in the list, but nonetheless included to the WADA monitoring program, and therefore, in principle, still to be searched for by the antidoping laboratories.

EXPERIMENTAL SECTION

Certified reference standards

Amphetamine, propylhexedrine, phenmetrazine, phendimetrazine, diethylpropion, fencanfamine, norpseudoephedrine (cathine), ephedrine, oxycodone, methylphenidate,

benzocaine, lidocaine, procaine, mepivacaine, tetracaine, bupivacaine and dibucaine were purchased from SALARS (Como, Italy); phentermine, methylamphetamine, ethylamphetamine, fenfluramine, MDA, MDMA, MDEA, MBDB, meperidine, benzphetamine, methadone, pentazocine, dextropropoxyphene and codeine were obtained from LGC Promochem (Teddington, UK); dimethylamphetamine, dextromoramide, fenproporex, mefenorex, clobenzorex, MTA, prolintane, were purchased from NARL (Pymble, Australia); Nikethamide, caffeine, methoxyphenamine, methylephedrine and diphenylamine were from Sigma-Aldrich, (Milano, Italy); Selegiline was from European Pharmacopeia (Strasbourg, France).

Instrumentation and consumables

GC/MS analyses were performed by a Hewlett-Packard 6890GC coupled with a Hewlett-Packard 5973 mass selective detector and equipped with a custom-made Supelco (MI, Italy) 5% phenylmethylsylicone capillary column (17m X 0.2 mm. i.d., 0,33 μm film thickness). GC injection port was set at 260°C in pulsed splitless mode (pulse pressure 50 psi for 0.5 min., purge time 2 min.); helium was used as carrier gas at a flow of 0.6 mL/min. The oven temperature was held at 90° C for 2', increased to 270°C at 7°C/min, then increased to 310 °C at 50°C/min, and held 2.8 min. The mass detector operated in electron impact at 70 eV in full scan, acquiring ions of m/z from 53 to 335. At the screening level, the possible presence in the sample of each substance considered in this study was monitored by checking the presence of diagnostic ions at the expected relative retention times.

100 μm polydimethylsyloxane (PDMS), Carbowax/divinylbenzene (CW/DVB), Carboxen/polydimethylsyloxane (CX/PDMS), polydimethylsyloxane/divinylbenzene (PDMS/DVB), divinyl-benzene/Carboxen/polydimethylsyloxane (DVB/CX/PDMS) and polyacrylate (PAC) fibers were supplied by Supelco and conditioned as prescribed.

Reference urines

Fifteen drug-free urines were obtained from laboratory staff. Methanolic stock solutions 1 mg/mL were used to prepare the spiked urine at a concentration of 1 μg/ml, to be diluted with blank urine to obtain working solutions at the desired concentration. Stock and working urines, as well as methanolic standard solutions, were stored at -20°C until use.

Urine pretreatment

0.5 mL of urine were diluted 1:1 in a 2 mL vial with a magnetic stirrer with 0.5 mL of carbonate buffer (pH 10) or distilled water (without pH adjustment); NaOH 2M was used to adjust pH for the pH 12 experiment. 10 μL of ISTD (diphenylamine 20 μg/ml) and 0.2 g of natrium chloride were then added to the sample. The fibre was dipped in this solution at 40°C

under stirring. The fibre was then directly inserted in the GC injection port and stripped at 260°C for 2 min. PDMS, CW/DVB, CX/PDMS, PDMS/DVB, DVB/CX/PDMS and PAC fibres were tested. Several parameters, influencing extraction efficiency and recovery, such as different sampling times (15, 20 and 30 min.) and different pH values of extraction (pH neutral, 9 and 12) were studied in order to optimize the method.

RESULTS AND DISCUSSION

Figure 1 shows the chromatograms of a positive reference urine, i.e. a negative urine spiked with the studied substances at the concentration of 500 ng/mL. Figure 2 reports the chromatograms of real positive samples: (a) a positive for methadone (estimated concentration 200 ng/mL); and (b) a positive for ephedrine at a concentration of 20.2 µg/mL.

PDMS/DVB coated fibre demonstrated the best sensitivity for all the analytes investigated. An alkaline pH was necessary to extract all the substances, and carbonate buffer at pH 10 showed the best performances in terms of recovery and fibre life. Adsorption time was optimized at 30 min, allowing an efficient extraction of all analytes and being coincident with GC runtime. A single fibre allowed at least 100 samplings without a significant fall in sensitivity. The described technique showed no carryover in the range studied for qualitative analysis (50-1000 ng/mL), while a carryover was observed for ephedrines and caffeine at concentrations higher than 5 µg/mL. For such concentrations it was necessary a preconditioning of the fibre at 260°C for 5 min.

All analytical parameters are reported in Table 1. The precision of SPME extraction was studied on five replicate analysis at 500 ng/mL, that is the WADA MPRL for stimulants. The results, expressed as intra-assay CV%, were comprised between 4.6 and 10.9, reflecting a precision acceptable for a qualitative analysis. Retention times (both absolute and relative) also showed a very low intra and inter-assay CV% (comprised between 0.0 and 0.6). The analytical recovery for each analyte was calculated at 500 ng/mL and expressed as the percentage over the theoretical sample concentration; it is comprised for all substances between 90 and 114%. Robustness of the method was evaluated by analyzing a spiked urine at 500 ng/mL using six PDMS/DVB fibres from different production batches. The same sample was also analysed once a week for 6 weeks: no significant differences were observed.

Compared to the reference method, generally performed by l/l extraction and GC/NPD analysis, SPME coupled with GC/MS shows an improved sensitivity and a better exploitation of human resources. Other advantages are the possibility of fully automate the procedure by a dedicated autosampler, the minimal use of solvents and the small volume of urine required.

Table 1

Substance	RRT	Diagnostic ions	LOD (ng/ml)	Curve equation	Linearity (R^2)
ISTD	1	169	---	---	---
Amphetamine*	0.20	91, 65, 120,134	50	y = 0.06x + 0.0153	0.990
Heptaminol	0.20	59, 56, 69, 113			
Fentermine*	0.27	58, 91, 134	50	y = 1.21x + 0.146	0.984
Propylhexedrine*	0.28	58, 55, 140	50	y = 2.35x + 0.0532	0.999
Methylamphetamine*	0.30	58, 91, 134	100	y = 1.13x + 0.158	0.986
Ethylamphetamine*	0.40	72, 91, 148	50	y = 1.69x – 0.166	0.990
Norfenfluramine	0.40	159, 184			
Fenfluramine*	0.41	72, 159, 109	50	y = 1.53 x – 0.063	0.999
Dimethylamphetamine*	0.42	72, 91, 148	50	y = 1.5 x + 0.118	0.996
Mephentermine	0.47	72, 91, 148			
Cathine*	0.55	77, 79, 117, 105	1000	y = 0.004x – 0.0088	0.994
Chlorphentermine	0.62	58, 125, 168			
Ephedrine*	0.64	58, 91	200	y = 0.219x + 0.60	0.990
Methoxyphenamine*	0.65	58, 9, 178	200	y =0.247 x + 0.042	0.977
Methylephedrine*	0.70	72, 77	50	y = 0.576x + 0.862	0.999
Selegiline*	0.72	96, 56	20	y = 1.59x – 0.279	0.980
Phenmetrazine*	0.75	71, 56, 177	100	y = 0.294 x – 0.007	0.995
Phendimetrazine*	0.78	57, 85, 191	50	y =0.321 x – 0.041	0.981
MDA*	0.80	135, 136, 77	100	y = 0.0545 x – 0.006	0.997
Diethylpropione*	0.83	100, 77, 72	50	y = 1.88 x – 0.303	0.985
MDMA*	0.88	58, 77, 135	100	y = 1.51 x – 0.257	0.982
Nikethamide*	0.89	106, 78, 177	100	y = 0.0654 x – 0.0118	0.984
MTA	0.92	138, 122, 91	100		
Pentetrazol	0.94	55, 138			
Benzocaine*	0.94	120, 165	50	y = 0.566x + 0.0097	0.987
MDEA*	0.95	72, 135, 91	50	y = 1.49 x – 0.161	0.994
Mefenorex*	0.96	120, 122, 91	50	y = 0.211 + 0.0013	0.986
Fenproporex*	0.98	97, 91, 56	50	y = 0.762 x – 0.129	0.980
MBDB	1.01	72, 135, 178	50		
Prolintane*	1.02	126, 91, 174	50	y = 2.93 – 0.46	0.993
Chrotethamide	1.04	86, 154			
Fencanfamine	1.09	215, 98, 186	20		
Methyilphenidate	1.09	84, 91, 115	50		
Furfurylamphetamine	1.09	81, 91, 138			
Mesocarb	1.11	91, 118, 65			
Chropropamide	1.11	168, 100			
Meperidine*	1.20	71, 247, 218	20	y =0.513x - 0.0223	0.998
Caffeine*	1.30	194, 109	200	y =0.0263x – 0.0084	0.995
Benzphetamine*	1.31	91, 148	20	y = 1.89 x - 0.20	0.994
Lidocaine*	1.37	86, 58	20	y =1.63x – 0.053	0.998
Clobenzorex*	1.46	125, 168, 91	20	y = 0.807x - 0.106	0.994
Amiphenazole	1.51	191, 121, 104			
Nafazoline	1.55	209, 141			
Procaine*	1.55	86, 120	50	y = 0.297x – 0.0242	0.997
Mepivacaine*	1.59	98, 70	20	y = 0.606x + 0.0129	0.997
Methadone*	1.68	72, 165, 294	20	y = 2.55x - 0.221	0.993
Pipradol*	1.69	84, 165, 56	200	y = 0.202x - 0.0618	0.930
Oxymethazoline	1.71	260, 245			
d-propoxyphene*	1.73	58, 91	20	y = 1.51x – 0.25	0.980
Tetracaine*	1.77	58, 71	20	y = 1.59x – 0.414	0.960
Pentazocine*	1.83	217, 285, 202	100	y = 0.0456x – 0.0082	0.970
Bupivacaine*	1.83	140, 84	20	y = 1.72x – 0.211	0.992
Codeine*	1.93	299, 229	200	y = 0.113x – 0.001	0.981
Oxycodone	2.07	315, 230, 258	200		
Amineptine	2.10	192, 315			
Dibucaine*	2.23	86, 116		y = 0.382x – 0.0461	0.999
Fenethylline	2.31	91, 250			
Dextromoramide*	2.37	100, 265	50	y = 0.306x - 0.0219	0.999
Strychnine	2.48	319, 334			
*: full validation completed, ISO17025 pending					

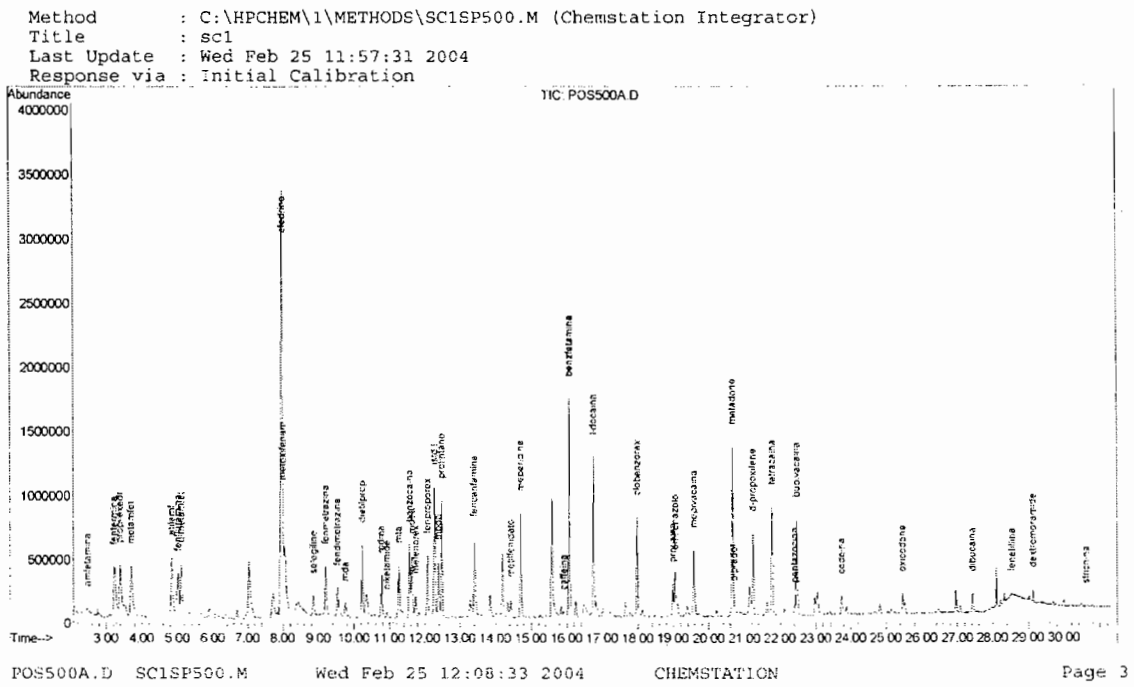

Figure 1. Chromatogram of a positive reference urine, spiked with the studied substances at a concentration of 500 ng/mL.

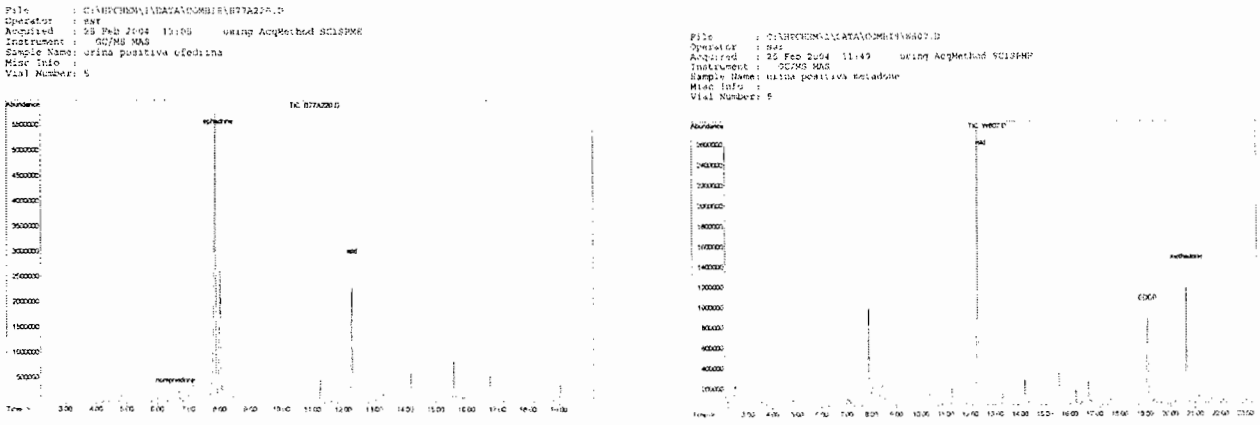

Figure 2. Chromatogram of real samples: urine samples positive for ephedrine (left) and for methadone (right).

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