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Fully Automatized Sample Preparation for Detection of 69 Doping-Related Substances

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Abstract

Sample preparation in a Doping Control Laboratory is a laborious work due to both the large number of samples to be treated and to the variety of compounds to be monitored. The diverse structural and chemical characteristics of the analytes entails different sample preparation procedures in order to purify, concentrate and prepare the samples prior to their analysis by the various instrumental techniques, including GC-MS, GC-MS/MS or LC-MS/MS. The automation of these sample preparation procedures is of great interest considering the saving in terms of time, security inside the laboratory and quality of results.
Any approach focused to the automation of sample preparation protocols needs specific equipment capable of carrying out the diverse mechanical tasks required for accomplishing the different analytical methodologies involved. Our laboratory has recently acquired an automated pipetting system for liquid-liquid extraction with shaking, heating, drying and crimping capabilities. In brief, the system was specifically designed for treating up to 96 samples in a sample preparation protocol which includes several heating and extraction steps. In this communication we present the successful automation of a method for the determination of 69 doping-related substances including anabolic agents, narcotics, anti-estrogenic substances, cannabinoids, diuretics and stimulants.

Introduction

Automation of sample preparation procedures in an Anti-Doping Laboratory is of great interest in terms of time saving, security and eventually the quality of the results obtained [1-3]. Our laboratory has recently acquired an automated pipetting system for liquid-liquid extraction with further capabilities including heating, shaking, drying and crimping. The first work has been focused on the automation of the method implemented in our laboratory for the determination of 69 doping-related substances in urine by GC-MS and GC-MS/MS analysis, whose sample preparation procedure includes enzymatic hydrolysis, liquid-liquid extraction and derivatization steps. A brief description of the equipment, as well as validation data including the recoveries obtained for all the substances are presented here.

Experimental

Reference materials were obtained from Sigma-Aldrich (St. Louis, MO, USA), NMI (Pymble, Australia), USP (Rockville, MD, USA), Atlanchim Pharma (Nantes, France), TRC (Toronto, Canada), Alltech (State College, PA, USA), Steraloids (Newport, RI, USA), European Pharmacopoeia (Strasbourg, France), AK Scientific (Union City, CA, USA), the World Association of Anti-Doping Scientists (WAADS) and Cerilliant (Round Rock, TX, USA). The rest of reagents and solvents were analytical grade. A standard stock methanolic solution containing all the compounds listed in Table 1 was prepared for validation purposes. Negative and positive urine samples were used in all the optimization and validation experiments. Positive samples consisted of negative ones spiked with the methanolic solution of standards at the concentrations depicted in Table 1.

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COMPOUND	CONC. (PPB)	RECOVERY AVERAGE (RSD)		COMPOUND	CONC. (PPB)	RECOVERY AVERAGE (RSD	
		AUTOM. (%)	MAN. (%)			AUTOM. (%)	MAN. (%)
4-OH-Testosterone	12	84.7(8.0)	93.4(3.5)	Metandienone M1 (Epimetendiol)	2	78.6(7.6)	89.5(9.1)
5α-Methyltestosterone	2	79.5(8.7)	90.7(9.9)	Metandienone M2 (6-OH-Dianabol)	2	93.4(4.2)	91.2(3.0)
5β-Methyltestosterone	2	80.3(8.0)	90.8(11.8)	Metandienone M3	10	69.6(6.8)	82.7(4.8)
6-Monoacetylmorphine	50	73.0(4.9)	71.9(4.1)	Metandienone M4 (17-Epimetandienone)	2	88.3(2.4)	93.6(6.4)
Aminoglutethimide	20	72.9(16.1)	75.1(22.5)	Metasterone M1 (3-OH-Metasterone)	5	80.1(7.4)	91.6(3.9)
Androstatrienedione	50	65.0(23.7)	70.8(55.4)	Metenolone	5	86.8(4.6)	93.9(4.2)
Bolasterone	5	84.7(6.9)	91.3(9.9)	Metenolone M1	5	84.9(3.6)	94.8(4.6)
Bolasterone M1	5	78.3(8.5)	90.7(10.3)	Methyldienolone	5	98.5(6.8)	91.4(15.0)
Boldenone	5	91.6(6.8)	88.6(9.4)	Methyl-1-testosterone	5	87.6(4.3)	94.2(9.9)
Boldenone M1	5	88.0(8.4)	92.3(4.9)	Mibolerone	5	89.0(3.7)	98.3(6.8)
Bromantane M1 (6-OH-Bromantane)	100	80.1(6.8)	91.9(4.4)	Morphine	50	21.9(3.2)	26.6(13.0)
Buprenorfine	5	75.6(5.0)	86.6(8.1)	Nandrolone M1 (19-Norandrosterone)	2	85.6(6.5)	94.0(7.2)
Buprenorfine M1 (Norbuprenorfine)	5	82.5(3.7)	62.6(14.4)	Nandrolone M2 (19-Noretiocholanolone)	5	87.8(5.5)	90.6(5.6)
Calusterone	5	83.1(4.9)	94.2(3.8)	Norboletone M2	5	73.0(5.9)	89.3(6.7)
Canrenone	200	80.7(2.7)	79.1(11.8)	Norethandrolone M1	10	80.2(6.6)	90.4(4.0)
Carphedon	100	10.1(7.2)	13.5(5.4)	Norethandrolone M2	5	81.2(6.9)	91.1(4.9)
Clostebol M1	5	83.5(6.1)	92.7(5.1)	Oxandrolone	5	44.3(9.2)	28.9(36.2)
Codeine	50	80.4(4.3)	74.3(3.4)	Oxandrolone M1 (Epioxandrolone)	5	43.5(8.9)	28.6(33.0)
Cyclofenil M2	20	91.1(4.9)	91.9(5.3)	Oxycodone	50	99.9(31.0)	109.7(23.3)
Danazol	5	87.1(12.6)	104.0(25.0)	Oxymesterone	5	84.9(6.1)	98.9(4.8)
Danazol M1 (Ethisterone)	5	84.1(4.2)	95.9(5.2)	Oxymorphone	50	65.8(30.8)	82.1(31.1)
Danazol M2	5	92.2(4.0)	98.8(5.2)	Parahydroxyamphetamine	100	42.6(13.1)	50.1(33.4)
Dehydrochlormethylte stosterone M1	2	98.5(6.6)	94.5(43.0)	Pentazocine	50	83.5(8.8)	91.4(3.9)
Drostanolone M1	5	79.8(7.5)	90.8(1.9)	Stanozolol M1	2	97.7(19.9)	89.6(44.9)
Estradienedione	5	99.0(5.1)	91.2(13.4)	Stanozolol M2	2	98.3(4.5)	103.1(28.1)
Estradienedione M1 (9(10)-Dehydronandrolone)	5	98.2(6.0)	88.3(10.6)	Tamoxifen M1	20	77.0(2.7)	87.8(9.7)
Fluoxymesterone	5	87.1(53.1)	85.4(36.7)	THC-9-COOH	15	87.4(2.7)	84.8(5.7)
Fluoxymesterone M2	5	82.9(6.1)	94.6(3.5)	Tibolone M1*	5	83.7(7.1)	97.2(7.4)
Furazabol	5	76.4(6.2)	89.4(16.2)	Tibolone M2	5	80.8(5.6)	92.8(4.7)
Furazabol M1	5	52.5(62.7)	81.2(29.3)	Tibolone M3	5	84.6(3.4)	96.3(5.4)
Hydrocodone	50	100.3(8.6)	97.5(25.7)	Trenbolone M1 (Epitrenbolone)	5	97.2(7.0)	101.7(41.6)
Hydromorphone	50	24.2(5.2)	33.7(22.2)	Zeranol	5	92.6(4.4)	93.3(5.5)
Letrozole M1	20	89.6(6.5)	90.2(3.5)	Zeranol M1 (Taleranol)	10	93.4(5.0)	92.0(4.1)
Mesterolone	5	85.9(6.0)	95.7(2.2)	Zilpaterol	5	39.1(8.9)	41.9(6.4)
Mesterolone M1	5	78.5(8.0)	91.4(9.3)				

Table 1: List of substances analyzed, concentrations in the positive urine samples and recoveries obtained in the automated and manual sample preparation.

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In routine work, the compounds listed in Table 1 can be extracted from urine samples following a manual three-step procedure which includes enzymatic hydrolysis, liquid-liquid extraction and derivatization, as depicted in Figure 1.
An automated liquid-liquid extraction system specifically designed for the needs of our laboratory, with additional shaking, heating, drying and crimping capabilities, was acquired from Zinsser Analytics (Frankfurt, Germany). The equipment consists of a workbench divided into different zones where the analytical tasks are carried out (see Figure 2). The layout integrates several modules among which a mobile gripper transports, picks up and drops off the different racks and tools, and four pipetting probes dispense and transfer solvents and reagents among the different areas. These probes self-load and discharge disposable tips thus avoiding potential cross-contamination.

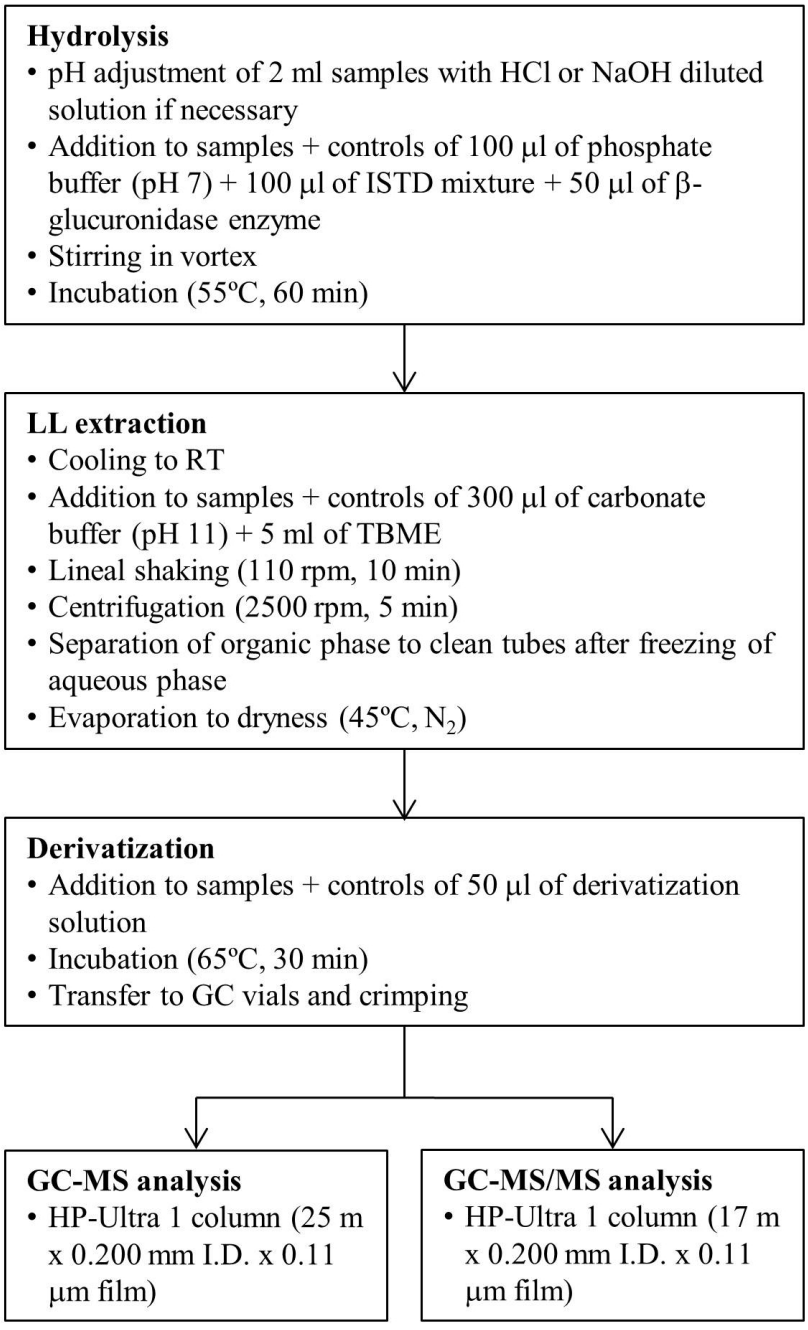

Figure 1: Flow chart of the manual sample preparation.

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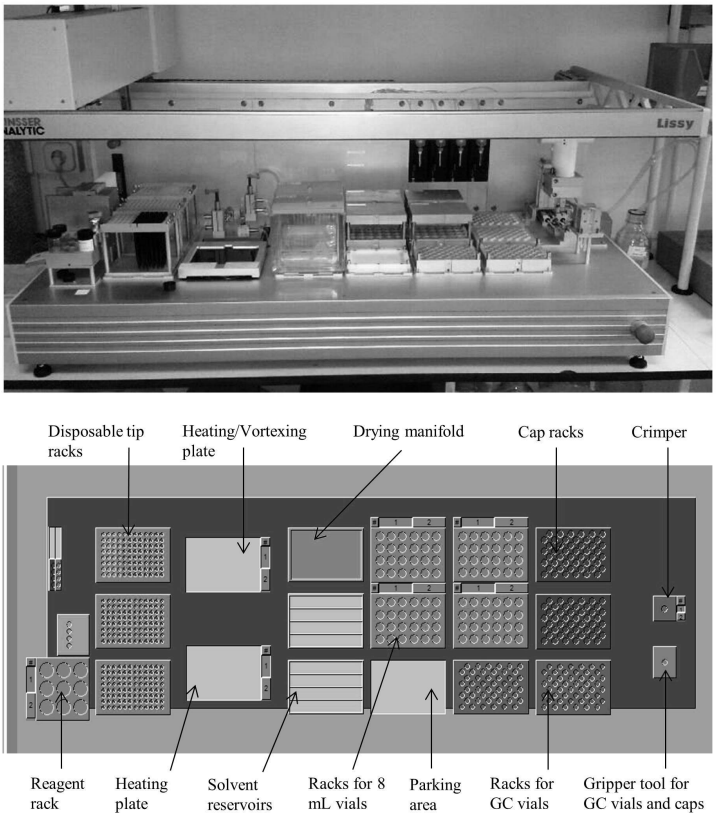

Figure 2: The Zinsser Lissy GXL automated sample preparation system.

All the samples were analysed by GC-MS or GC-MS/MS (Agilent Technologies, Palo Alto, CA, USA). The validation was approached as a comparison between the results obtained by applying the manual sample preparation method and the automated one; therefore the instrumental methods of detection were maintained with respect to those established in our laboratory [4].

Results and Discussion

The automation of the process depicted in Figure 1 required specific previous studies in order to adapt the protocol to the characteristics of the equipment, in particular regarding the liquid-liquid extraction step. Figure 3 shows the complete flow chart of the automated method with the values selected for the different parameters after optimization (shaking speed and time, waiting time for phase separation, number of extractions required, temperature and drying time).
Selectivity. Four 2 ml negative urine samples were analysed (five replicate experiments) according to the automated protocol finally selected. Analytical results were then compared to those obtained in the manual sample preparation. No interfering peaks were observed that could affect the performance of the method. An additional experiment conducted with samples spiked at twenty times the concentrations indicated in Table 1 also demonstrated the absence of cross contamination or carry-over issues.

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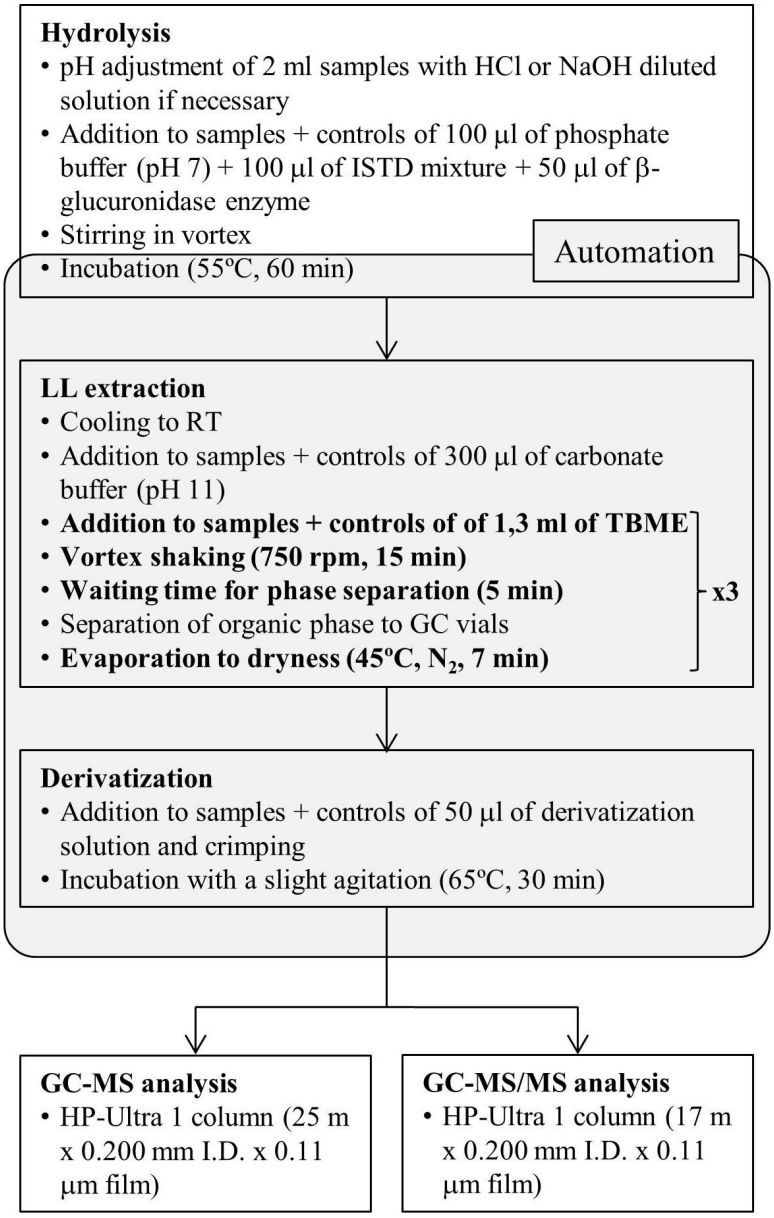

Figure 3: Flow chart of the automated sample preparation. Steps automated are inside the grey square. Steps that required optimization prior to the validation study are shown in bold letters.

Extraction efficiency. Recoveries were determined by extracting pairs of 2 ml distilled water samples: one of them was spiked prior to the preparation procedure at four times the concentrations indicated in Table 1. On the other hand, a 2 ml distilled water sample was prepared together with a GC vial spiked similarly and dried, indicating full recovery. Samples were then extracted in quintuplicate by using the automated and manual sample preparation methods. Extraction efficiency was calculated as mean percentages of the full-recovery samples (see Table 1). Overall, the average recoveries observed in the automated sample preparation method compared well with those obtained in the manual one.
Sensitivity. 2 ml urine positive samples (negative samples spiked at the concentrations shown in Table 1) were extracted in triplicate according to the selected automated procedure. Analytical results were then compared to those obtained in the manual sample preparation. Overall, successful determination was achieved for all the analytes included in the study.

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Conclusions

A fully automated sample preparation method for the analysis of 69 doping-related substances was developed by using an automated liquid handling system specifically designed for the laboratory. The method allows preparation of up to 96 urine samples in one simple experiment with just 1-hour time of previous intervention by an operator. The recoveries and sensitivities obtained with the automated method were similar to those obtained with the manual one and fulfilled the requirements of WADA for all the substances involved [5]. The competence of the automated protocol was also tested in terms of selectivity, contamination and carry-over effects. The described method is at present suitable for routine analyses and is being applied daily in our laboratory with high sample throughput.

References

[1] Soriano C, Muñoz-Guerra J, Carreras D, Rodríguez C, Rodríguez AF, Cortés R. (1996) Automated analysis of drugs in urine. *J. Chromatogr. B* **687**, 183-187.
[2] Haber E, Muñoz-Guerra JA, Soriano C, Carreras D, Rodríguez C, Rodríguez AF. (2001) Automated sample preparation and gas chromatography-mass spectrometry analysis of urinary androgenic anabolic steroids. *J. Chromatogr. B* **755**, 17-26.
[3] Vuckovic D, Cudjoe E, Hein D, Pawliszyn J. (2008) Automation of solid-phase microextraction in high-throughput format and applications to drug analysis. *Anal. Chem.* **80**, 6870-6880.
[4] Massé R, Ayotte C, Dugal R. (1989) Studies on anabolic steroids: I. Integrated methodological approach to the gas chromatographic-mass spectrometric analysis of anabolic steroid metabolites in urine. *J. Chromatogr. B* **489**, 23-50.
[5] World Anti-Doping Agency. Technical Document TD2013MRPL: Minimum required performance levels for detection and identification of non-threshold substances. International Standard, Montreal (2013) http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/Technical_Documents/WADA-TD2013MRPL-Minimum-Required-Performance-Levels-v1-2012-EN.pdf (access date 29.04.2013).

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