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An Automated SPE-LC/MS Method Permits a Highly Selective Analysis of  
Anabolic Steroids, Beta-Blocking Agents and Stimulants  
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## **An Automated SPE-LC/MS Method Permits A Highly Selective Analysis Of Anabolic Steroids, Beta-Blocking Agents And Stimulants**

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### **Introduction**

The list of the World Antidoping Agency (WADA) groups prohibited substances according to pharmacological activity, due to their common chemical structure. Each year new compounds are listed that must be tested by the Antidoping Laboratories, thus extending the range of chemical structures, and obliging them to increase or modify the number of procedures they perform. This study aimed at developing a single flexible method to facilitate the inclusion of new compounds and permit screening for different families of compounds with a broad range of chemical properties (acid, basic, and neutral). The method's high level of selectivity and sensitivity satisfies WADA's minimum required performance limits (MRPL).

### **Experimental**

Urinary aliquots from a modafinil excretion study<sup>1,2</sup> (doses 50mg of Modiodal®, after 3h administration) were spiked with carvedilol, atenolol, salbutamol, gestrinone, tetrahydrogestrinone (THG), betamethasone, fluocinole and 6 $\alpha$ -methylprednisolone.

Bethametasone, fluocinole acetonide and 6 $\alpha$ -methylprednisolone were obtained from Sigma-Aldrich (St. Louis, USA), salbutamol from British Pharmacopoeia (London, UK), atenol from United States Pharmacopeial (USA), THG from National Analytical Reference Laboratory (Pymble, Australia). The other standards were obtained from medicaments: gestrinone (Nemestran®, Roussel Iberica, Spain), carvedilol (Coropres®, Roche, Spain).

Samples were extracted and analysed with an automatic sample preparative station (Gilson Aspec XLi) hyphenated to an LC-MS system, LCQDuo (Thermo Finnigan), equipped with an electrospray ionization (ESI) interface.

*Extraction procedure:* 100 $\mu$ l of pH 7 phosphate buffer, 25 $\mu$ l of E.Coli  $\beta$ -glucuronidase and 40 $\mu$ l of the internal standard, timolol (50ng/ml), were added to each 2ml aliquot. Each tube

was heated for 1 hour at 55°C and after hydrolysis the pH was adjusted to 2-3 with a few drops of diluted HCl and then centrifuged for 5 minutes at 2500 rpm.

The OASIS® MCX 1cc cartridges (30 mg sorbing mass) were conditioned with 500µl MeOH and later with 500µl water both at a 3ml/min flow. Analytes were loaded by passing 2ml of the urine at a 1ml/min flow. Next, cartridges were washed with 2ml of 0,1N HCl and 500µl water/MeOH 60:40 at a 3ml/min flow. Finally, a first elution was done with 2ml of methanol (elute A) and a second one with 2ml a mixture of dicloromethane/methanol (20/80) with 1% of NH<sub>4</sub>OH (elute B) both at a 1ml/min flow.

The hydrophobic (elute A) and the basic (elute B) extracts were evaporated to dryness under nitrogen and reconstituted in a 200 µl acetate ammonium 10mM buffer.

*Instrumental conditions:* Table 1 summarises the main LC-ESI-MS parameters and the retention time and the ions monitored for each compound<sup>3,4,5</sup>.

## **Results and discussion**

### a) Method optimisation:

Oasis® MCX cation-exchange cartridges contain a porous resin that through different interactions (hydrophobic, hydrogen bonding and cation-exchange interactions) makes it possible to link acid, hydrophobic and basic analytes. This dual retention mechanism permits fractionated and much more specific elution.

Many parameters have been checked to optimise the procedure; sample pH, percentage of methanol in cleaning solution, elution steps, etc. The percentage of NH<sub>4</sub>OH in the elution mixture plays an important role in reaching a cleaner B extract with high recovery, as shown in Fig.1. There is quite a strong relation between basic compounds recovery and percentage of NH<sub>4</sub>OH with 1% ensuring total elution. A higher percentage of NH<sub>4</sub>OH does not enhance the recovery and makes it more difficult to dry the extract.

### b) Testing the extraction for a high throughput of compounds:

With most of the routine liquid-liquid extraction procedures, acid and basic drugs cannot be extracted in a single step and automation is difficult. Polar and apolar compounds cannot be extracted at the same time using a C-18 solid phase extraction procedure; however, they can with OASIS® MCX SPE cartridges (see Figs. 2 and 3).

### c) Validation results:

*Selectivity:* Spiked urine with acid (acid metabolism of modafinil), basic (carvedilol) and neutral compounds (anabolic steroids and corticosteroids) was extracted with this

methodology. Acid and neutral compounds were eluted only in the methanolic elution (elute A). Basic compounds elute in the basic elute (elute B). Elute A was checked for basic compounds, and elute B was checked for acid and neutral compounds. Each elute proved to be selective for a specific class of compound (see Fig. 3).

*Limits of detection (LOD):* To measure the LOD for the tested compounds, negative urine was spiked at a concentration of 50 ng/ml for corticosteroids, THG and gestrinone; and 100ng/ml for carvedilol. 10 aliquots of this control and negative urine were extracted and analysed for a time period that covered different degrees of performance for the instrumentation. Three times the standard deviation of noise in the negative urine was used to estimate the theoretic LOD. They were then checked experimentally as shown in Fig. 4. In all cases MRPL were complied with.

*Carry over:* To evaluate possible carry over between samples, negative urine was spiked with 5mg/ml of carvedilol, 300 ng/ml of corticosteroids, 100ng/ml of THG, 10 times higher than the MRPL. Then, some aliquots were extracted and analysed by LC-MS. No traces of carry over were observed in any case.

*Precision:* 34 aliquots of a spiked sample (100ng/ml carvedilol and corticosteroids, 75ng/ml THG and gestrinone) were processed. Fig. 5 shows the means and the standard deviation for the ratio area compound/area ESTD (20µl Timolol 50µg/ml) of each compound.

## **Conclusion**

The results obtained indicate the potential of this technique as an optimum procedure for screening a broad range of compounds. The dual retention mechanism of the resin permits, with a 2 ml aliquot, screening for acid (e.g. modafinil), neutral (e.g. anabolic steroids, corticosteroids) and basic (beta blocker agents, beta-2 agonist, stimulants) compounds. By hyphenating the preparative station Aspec XLi (GILSON) to a LC/MS system a robust, quick, selective and flexible method is set up that allows inclusion of new analytes of very different chemical properties into the screening procedure.

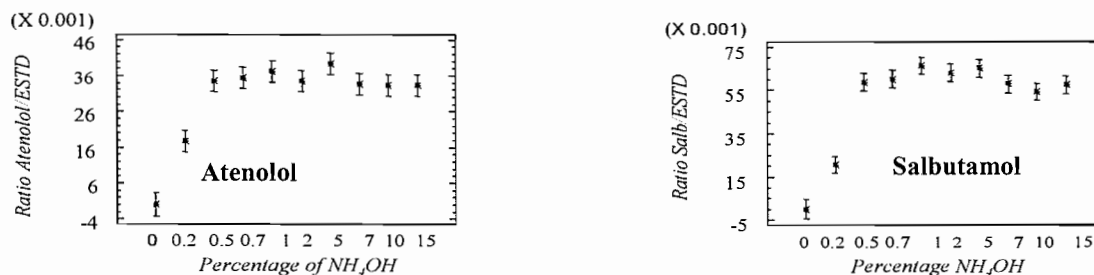
## **References**

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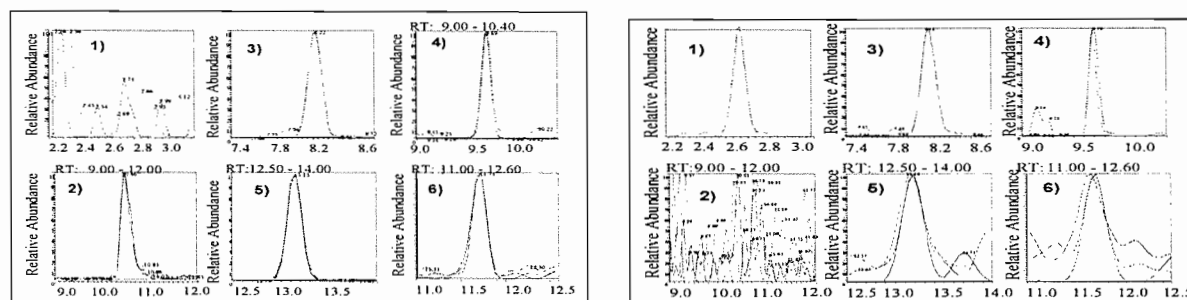
## Figures and tables

**Table 1.** LC- MS conditions.

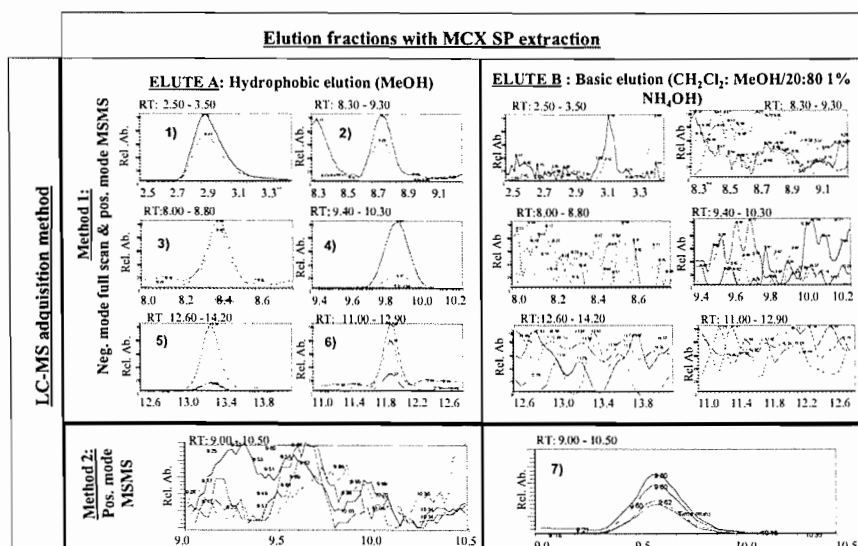
HPLC CONDITIONS						
Column:	Inertsil 3-ODS (Varian) 150mm x 3.0mm 3mm					
Flow rate:	400 $\mu$ l/min					
Volume inj:	3 $\mu$ l					
Solvents:	A: 10mM $\text{NH}_4\text{CH}_3\text{CO}_2$ solution, B: Acetonitrile					
Gradient:	0-4min 30% B; 8-13min 90%B; 14-21 min 30%B					
MS CONDITIONS						
Spray voltage:	4.58 Kv.	Capillary Temp:	300°C.			
Method acquisition	Time (min)	Acq.n mode [mass range]	Parent Ion / (Collision Energy)	Compound	Retention time	Ions monitored
Method 1 (for elute A)	0-11	- cESI Full ms [155-400]		Acid met. modafinil	2.96	273; 167
				6 $\alpha$ -methylprednisolone	8.49	433; 343
				Bethametason	8.84	451; 361
				Fluocinolone	10.00	511; 431; 451
	11-21	+c ESI Full MS2 [85-400]	309/ (34.50)	Gestrinone	12.03	281; 291; 241
			313/ (32.00)	Tetrahydrogestrinone	13.35	295; 225; 239
Method 2 (for elute B)	0-21	+c ESI Full MS2 [110-500]	407/ (32.00)	Carvedilol	9.63	283; 222; 224



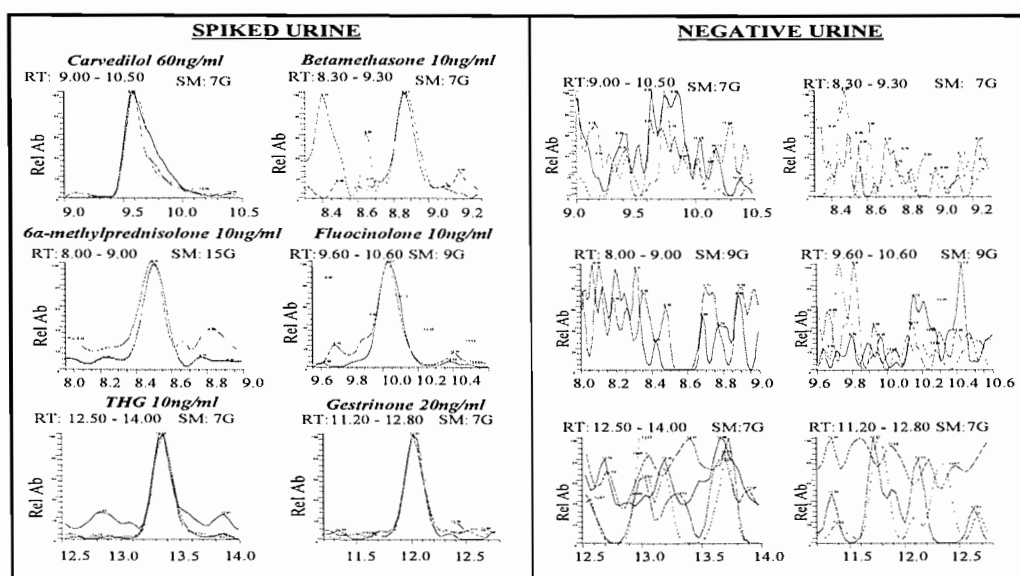
**Figure 1:** Recovery of atenolol and salbutamol versus percentage of  $\text{NH}_4\text{OH}$  in the elute mixture. Each point represents 5 replicates.



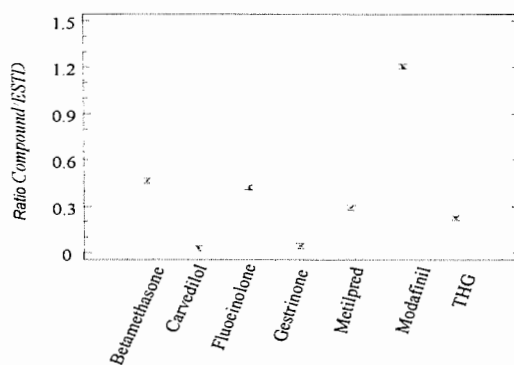
**Figure 2:** Left: generic liquid-liquid extraction at basic pH; right: generic C-18 SP extraction of 1) acid metabolite of modafinil, 2) carvedilol (basic compound), 3) 6 $\alpha$ -methylprednisolone, 4) fluocinolone, 5) THG, 6) gestrinone (3, 4, 5 and 6 are neutral compounds). Elutes of both extractions were analysed using the acquisition methods described in table 1.



**Figure 3:** Selectivity of the extraction: Elutes A and B were analysed using the acquisition methods 1 and 2. 1) Acid metabolite of modafinil, 2) betamethasone, 3) 6 $\alpha$ -methylprednisolone, 4) fluocinolone, 5) gestrinone, 6) THG, 7) carvedilol.



**Figure 4:** LOD checked experimentally.



**Figure 5:** Precision: Average means versus standard deviation values for the ratio area compound/area ESTD. Each point represents 32 aliquots.