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Possibilities of SIM/scan analysis in doping control

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Introduction

GC-MS plays an important role in doping control. Following proceedings of previous workshops the products of Agilent Technologies are widespread in doping control laboratories. Since the introduction of the first GC-MS equipment evolution has resulted in the availability of benchtop instruments, monolithic quadrupoles, gold plated quadrupoles and inert sources. In 2006, a new instrument was marketed (MSD 5975) with several new features compared to previous series. Besides the introduction of a window on the mass spectrometer allowing accurate column positioning, the most important improvement was the introduction of high performance electronics allowing a faster data transfer and a higher scan rate. In addition, the high data transfer permits the alternative acquisition of SIM and scan data.

At present analytical methods in doping control using gas chromatography are used for the detection of anabolic androgenic steroids, narcotic agents and stimulants. While in the past GC-NPD was often used to determine volatile nitrogen containing stimulants such as amphetamine [1-3], a comprehensive GC-MS screening method is now available for the simultaneous detection of narcotic agents and stimulants using MSTFA derivatisation [4]. Additionally a second analytical method is used to detect anabolic steroids in urine (traditionally called extraction procedure IV in doping control laboratories)[5, 6]. While the former is performed in the scan mode the latter uses selected ion monitoring (SIM).

As the new generation of analytical equipment allows for the simultaneous acquisition of SIM and scan data a combination of the currently used analytical methods could result in an increased productivity and reduce the total analytical run time. Therefore the possibilities of SIM/scan in doping control was evaluated.

Experimental

GC/MS conditions

The GC/MS analysis is carried out on an Agilent 5975 mass spectrometer directly coupled to an Agilent 6870 gas chromatograph equipped with a J&W-Ultra 1 column with a length of 17 m, internal diameter of 0.2 mm and a film thickness of 0.11 µm. The GC is operated in constant flow mode at a flow rate of 0.6 ml/min. The oven temperature is as follows:
70°C (0 min) → 90°C/min → 100°C (5 min) → 30°C/min → 180°C (0 min) → 3°C/min → 232°C (0 min) → 40°C/min → 310°C (3 min).
Half a microliter is injected in the splitless mode.
The mass spectrometer is operated in the SIM/scan mode. Scan parameters are as follows:

- 2.3 min → 10 min: m/z 50 – 390 (4.15 scans/s)
- 10 min → 16.5 min: m/z 50 – 525 (3.06 scans/s)
- 16.5 min → 25.78 min: m/z 50 – 650 (2.46 scans/s)

In the SIM mode 15 groups were created with a dwell time for the individual ions of 10 ms (scan rate 3.42 – 24.32 scans/s).

Extraction

Extraction is performed with 4 ml of urine divided in aliquots of 3 and 1 ml for the extraction of the conjugated and free components, respectively.
One ml of phosphate buffer (pH 7), 50 µl of β-glucuronidase (E. coli K12) and 50 µl of the internal standard 17α-methyltestosterone (2 µg/ml) are added to 3 ml of urine after which the the sample was hydrolysed overnight at 42°C. Extraction was performed with 5 ml of diethylether after the hydrolysate was made alkaline with 1 ml ammonium buffer (pH 9.5). After rolling for 20 minutes and centrifugation (1200 g, 5 min) the organic layer was separated and evaporated under oxygen free nitrogen at 40°C.
To the other aliquot of 1 ml urine, 50 µl of the internal standard cyclopentamine (100 µg/ml), 1 ml of KOH (5 M), 1 g of NaCl and 1 ml of TBME were added. After rolling for 20 min and centrifugation (1200 g, 5 min) the organic layer was added to the residue of the extraction performed at pH 9.5 and evaporated under oxygen free nitrogen at room temperature. The final residue was derivatised with 100 µl MSTFA/NH_4I/ethanethiol (640/1/2 ; v/w/v) for 1 h at 80°C.

Results and discussion

As a result of the introduction of high performance electronics on the Agilent 5975 faster data transfer is achieved and a scan rate up to 10000 units per second can be reached. Consequently, more data points can be acquired in a chromatographic peak allowing improved peak integration. In addition the MSD 5975 allows for the alternative acquisition of SIM and scan spectra in one analytical run. While SIM offers a higher sensitivity, interfering peaks can now be identified using the scan trace and commercial or self developed libraries. According to Agilent the simultaneous SIM/scan acquisition can be performed without loss of sensitivity in the SIM trace. This was tested by the analysis of 17α-methyltestosterone-bis-TMS, the internal standard routinely used in screening IV. As can be seen from Figure 1 SIM/scan results only in a small loss of sensitivity.

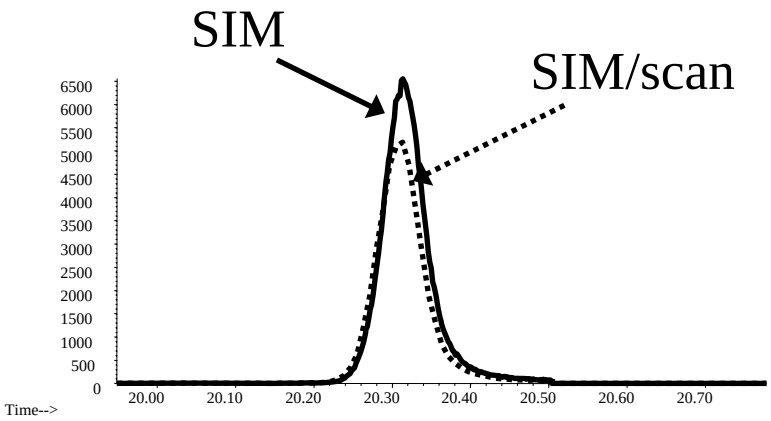

Figure 1: acquisition of 17α-methyltestosterone-bis-TMS in SIM mode compared to SIM/scan mode.

For doping control purposes the combination of SIM and scan could be useful. Unknown peaks in SIM traces can be identified and it could also allow for the combination of current methodology. This may not result in a loss of sensitivity and selectivity. In addition to these requirements the combined analytical method should have a reasonable analytical runtime to increase productivity.

The first analytical problem is derivatisation. While in current methodology narcotics and stimulants are derivatised using MSTFA, anabolic steroids are derivatised with MSTFA/NH_4I/ethanethiol (320/1/2 ; v/w/v) to create TMS-enol-ethers. The latter derivatisation mixture however results in bad chromatography and decreased sensitivity for narcotic agents such as morphine in comparison to a 50% diluted mixture (i.e.

MSTFA/NH_4I/ethanethiol 640/1/2 ; v/w/v) (Figure 2a). In addition, this diluted mixture does not influence peak shape and sensitivity (Figure 2b) for anabolic steroids.

At present anabolic steroids are analysed within a 22.3 min run using a fast increase in temperature to 180°C after which anabolic steroids are chromatographically separated at a temperature rate of 3°C/min. For narcotics and stimulants on the other hand, temperature needs to be isothermal during the first part of the chromatographic run to avoid the loss of the very volatile substances such as methylamphetamine in the solvent front. Afterwards, temperature is increased at a rate of 20 °C per minute resulting in an analytical run of 18.4 minutes.

In order to combine both methods, the isothermal part was retained after which temperature was increased to 180°C. Hereafter the slow gradient of 3°C per minute allowed for the separation of the anabolic agents. In total, the runtime is 25.8 minutes, 3.5 minutes longer than for the current screening IV.

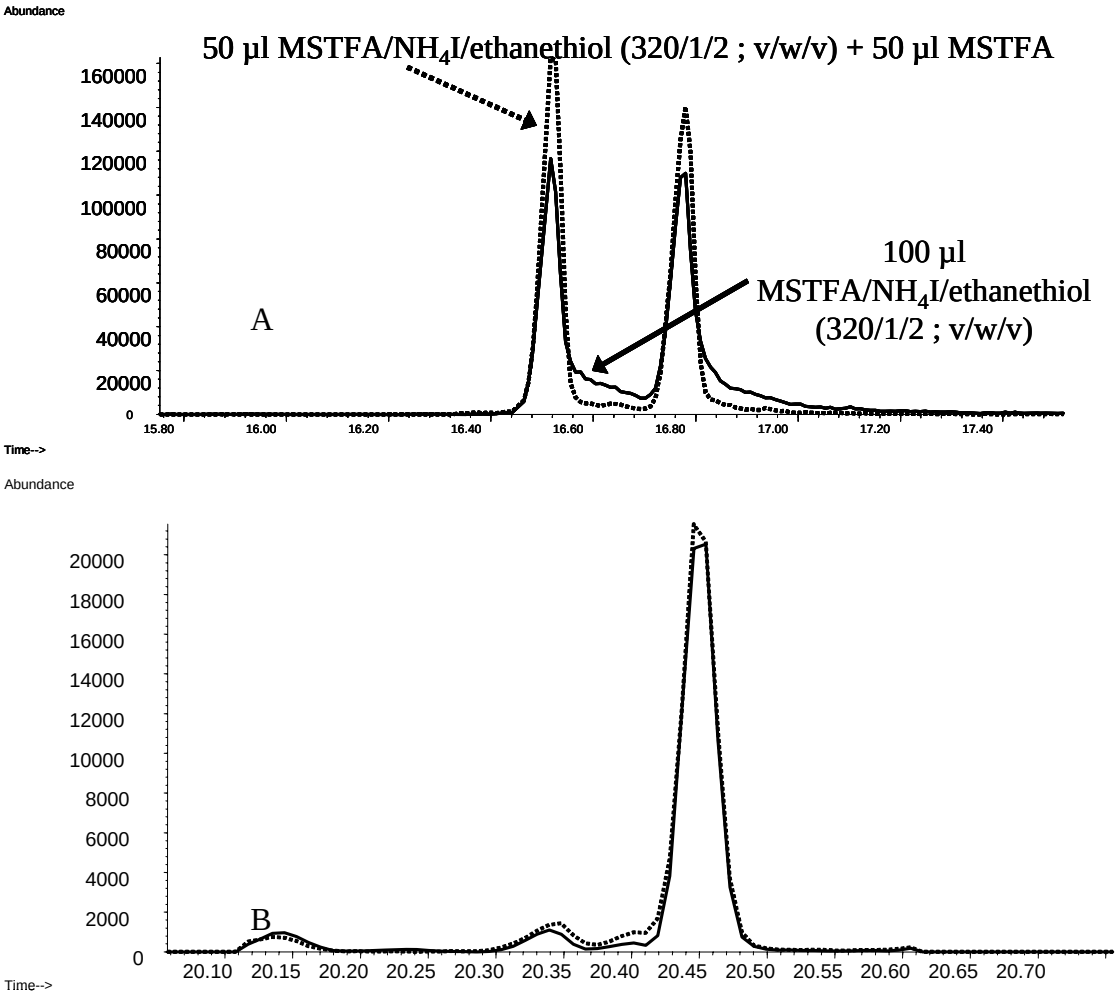

Figure 2: Comparison of MSTFA/NH_4I/ethanethiol (320/1/2) and MSTFA/NH_4I/ethanethiol (640/1/2) as derivatisation agent for morphine and hydromorphone (A) and 17α-methyltestosterone (B).

In order to obtain as many scans as possible scan parameters were divided into three groups. During the first 10 minutes only low weight components such as stimulants elute and the mass range is restricted to m/z 390, while later during the chromatographic run this mass is increased to 525 and 650 respectively.
In the SIM trace, 15 groups are created with a maximum of 25 ions in one group. Dwell times were set at 10 millisecond resulting in SIM scan rates between 3.42 and 24.32 scans/s. The dwell time could be decreased resulting in more scans cycles, but this might decrease the sensitivity.
The mass spectrometer is alternatively operated in the SIM and scan mode and the combined SIM/scan rate can be calculated as follows:

$$1 \Big/ \left(\left(1 \Big/ SR_{SIM} + 1 \Big/ SR_{scan} \right) \times 1.05 \right)$$

In this method, the lowest total scan rate is 1.36 scans/s resulting in about 9 data points during a normal GC peak. Compared to the current anabolic screening method this is 0.3 scans per second higher.
To allow for the extraction of the stimulants the procedure was divided in extractions at pH 9.5 and 14, similar to the combined screening method for narcotics and stimulants [4]. For the conjugated components 3 ml of urine was hydrolysed overnight permitting the complete hydrolysis of morphine [4] and norandrosterone. After the hydrolysis, ammonium buffer was used instead of a solid carbonate buffer and several organic solvents were tested as well. For anabolic steroids and narcotic agents ethyl acetate provided the best results but very high interferences of urea and glycerol in the first part of the chromatographic run hampered the detection of the volatile stimulants. The combination of dichloromethane and methanol as currently used for conjugated narcotics and stimulants gave bad results for the anabolic steroids. The best option was the use of diethyl ether although this resulted in the loss of benzoylecgonine, the urinary marker of cocaine. Nevertheless, diethyl ether was chosen as the extraction solvent at pH 9.5.
Extraction at pH 14 is as usual with tertiarymethylbutyl ether.
This combined method allows for the detection of 158 components. These include all anabolic steroids, both exogenous and endogenous, narcotic agents, stimulants, agents with anti-estrogenic activity and beta-agonists present in our current GC-MS screening methods.

The validation of this analytical method was divided in a qualitative and quantitative part. The qualitative validation was performed on 10 different negative urines spiked at three different levels (MRPL/2, MRPL and 2xMRPL). The LOD was defined as the lowest concentration for which a score of 10/10 was observed. For 4 components no satisfying results could be obtained at the MRPL level. These substances are benzoylecgonine, heroin, fluoxymesterone tetrol and zilpaterol. Benzoylecgonine could not be detected. Heroin was not detected, but its major metabolite, 6-mono-acetylmorphine, was validated at 100 ng/ml or half the MRPL level. The abuse of fluoxymesterone can not be detected using this method if 9α-fluoro-17α-methyl-androst-4-ene-3α,6β,11β,17β-tetrol is monitored. However, the method validation for 9α-fluoro-18-nor-17,17-dimethyl-androsta-4,13-dien-11β-ol-3-one, another metabolite of fluoxymestrone [7], was successful at the MRPL level of 10 ng/ml. For zilpaterol, a score of 10/10 could be obtained at 20 ng/ml. All other component could be detected at or below the MRPL level set by WADA [8]. Selectivity was tested by the analysis of a reference mixture of exogenous corticosteroids which did not result in the detection of interferences at the retention times of the screened components. Analysis of the 10 different negative urines to evaluate specificity resulted in the detection of an interference for 17α-trenbolone and 7β-OH DHEA eluting within an interval of 0.1 minutes. Taking a closer look to the scan trace revealed that this interference originated from nalorphine, the internal standard used for the narcotic agents. After the removal of this internal standard both components could be validated at the MRPL level of 10 ng/ml.

Besides the qualitative method validation a quantitative validation was carried out for the endogenous anabolic steroids, morphine, tetrahydrocannabinol and salbutamol. As shown previously[4], quantification of ephedrines using this method is not possible due to the formation of multiple derivatives.

Linear calibration curves could be obtained with correlation coefficients higher than 0.997 (Table 1) for all endogenous steroids. The trueness on three different concentrations of the calibration curves was always within the allowed margin of 20 % just as the repeatability and reproducibility of which the margins are concentration dependant according to the Horwitz equation [9].

For morphine a linear calibration curve was obtained with both internal standards (nalorphine and 17α-methyltestosterone). This shows us that nalorphine can be removed as internal standard which also solves the problem associated with 17α-trenbolone and 7β-OH-DHEA. Only the reproducibility for morphine exceeds the allowed margins. However, a safety margin

of 50 % could reduce the number of confirmation procedures without the risk of a false negative result.

For THC and salbutamol no linear calibration curves could be obtained. The addition of a deuterated analogue might be an answer to this problem.

Table 1: Results of the quantitative method validation.

Component	Calibration range (ng/ml)	R^2	Trueness (%)	Repeatability (%)	Reproducibility (%)
Androsterone	300 - 4800	0.997	81.95 - 106.08	1.78 - 10.85	9.90 - 11.92
Etiocholanolone	300 - 4800	0.998	82.10 - 103,19	2.05 - 8.67	7.68 - 18.33
Testosterone	25 - 400	0.998	85.70 - 107.58	1.23 - 8.00	5.23 - 8.11
Epitestosterone	25 - 400	0.999	93,32 - 111.47	1.77 - 5.57	5.57 - 12.57
5α-Androstane-3α,17β-diol	25 - 400	0.998	91.59 - 106.99	2.84 - 14.18	11.12 - 14.06
5β-Androstane-3α,17β-diol	25 - 400	0.999	89.49 - 102.40	2.29 - 9.39	8.94 - 16.62
5α-Androstane-3β,17α-diol	25 - 400	0.998	95.01 - 105.95	2.05 - 9.82	6.23 - 8.01
DHEA	25 - 400	0.999	86.57 - 104.55	1.30 - 7.20	7.46 - 13.33
DHT	25 - 400	0.999	90.75 - 111.77	2.44 - 9.37	5.07 - 15.03
4-androstene-3,17-dione	25 - 400	0.999	87.48 - 109.08	0.80 - 7.53	10.17 - 14.83
11β-OH-androsterone	250 - 4000	0.998	81.88 - 112.00	1.59 - 12.71	9.60 - 17.60
11β-OH-etiocholanolone	250 - 4000	0.999	89.79 - 108.44	2.21 - 9.34	5.21 - 17.46
morphine	250 - 2000	0.985	92.69 - 101.60	2.39 - 10.57	22.77 -37.86
THC	5 - 100	/	/	/	/
salbutamol	250 - 500	/	/	/	/

Conclusion

Following these results it can be concluded that the combination of SIM/scan can be used in doping control analysis. Using this combined method all components included in the former screening methods can be detected in one single analytical run without loss in sensitivity.

Acknowledgements

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