Journal of Chromatography A, 1217 (2010) 4749–4752

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Short communication

The use of a simple backflush technology to improve sample throughput and system robustness in routine gas chromatography tandem mass spectrometry analysis of doping control samples

B.P. Gray*, P. Teale

HFL Sport Science, Quotient Bioresearch, Newmarket Road, Fordham, Cambridgeshire CB7 5WW, UK

ARTICLE INFO

Article history:
Received 26 February 2010
Received in revised form 21 April 2010
Accepted 3 May 2010
Available online 6 May 2010

Keywords:
Backflushing
Gas chromatography
Doping control
Anabolic steroids
Equine

ABSTRACT

A simple, low cost system for the backflushing of capillary gas chromatography (GC) columns has been investigated and integrated into a method for the detection of anabolic steroids in equine urine. The modification to the method was simple to make and quick to setup and optimize. The use of backflushing technology was found to offer significant benefits in terms of sample throughput and improved system robustness.

1. Introduction

The screening of equine doping control samples for the presence of anabolic steroid abuse has been routinely performed in anti-doping laboratories around the world for many years [1–7]. Screening has historically been performed on urine samples, with gas chromatography utilizing selected ion monitoring (SIM) as the technique of choice. With the continuing drive towards lower limits of detection there is a requirement for more sensitive and specific methods. The implementation of triple quadrupole technology has led to improved detection limits and increased specificity due to the superior discrimination power offered by selected reaction monitoring (SRM) over SIM.

Recently we transferred an existing screen for anabolic steroids in equine urine from single to triple quadrupole technology. In addition to widening the coverage of the screen to include more steroids and their metabolites the main aims of the transfer were to improve the sensitivity of the screen, simplify data checking by improving specificity and to reduce sample turn around time by improving sample throughput.

In a typical gas chromatography analysis there is an extended hold period at the end of the run, after the last analyte of interest has eluted, during which the oven temperature is increased, usually for a period of 2–10 min. The purpose of this high temperature hold time is to remove late eluting or 'high boiling' matrix components from the analytical column. These compounds otherwise accumulate in the analytical column from one injection to the next and could cause problems with chromatography and method sensitivity. In some cases the late eluting compounds elute during the subsequent injection, potentially at a point in the analysis where analytes of interest are also eluting. There is also the additional problem of the late eluting components entering the ion source of the mass spectrometer. Whilst the use of MSMS will limit the potential for false identifications resulting from this 'breakthrough' of late eluting peaks from one run to another the presence of these components increases fouling of the ion source and ultimately leads to reduced analyte response and increased requirement for source cleaning. The use of a high temperature hold time at the end of an analytical run also increases total cycle time and hence impacts on sample throughput.

Column backflushing offers potential advantages in terms of reduced cycle times, enhanced column life and reduced maintenance of mass spectrometer components [8]. Backflushing systems are available as a system add-on from some gas chromatograph manufacturers and generally comprise of a solid state capillary flow device installed in the GC oven, an additional electronic pressure controller and software to calculate and control gas pressures. Whilst offering an easy and effective method of setting up backflushing, these systems add considerably to the cost of the initial package and are not available for all commercially available MS

* Corresponding author. Tel.: +44 01638 724272.
E-mail address: bgray@hfl.co.uk (B.P. Gray).

0021-9673/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2010.05.004

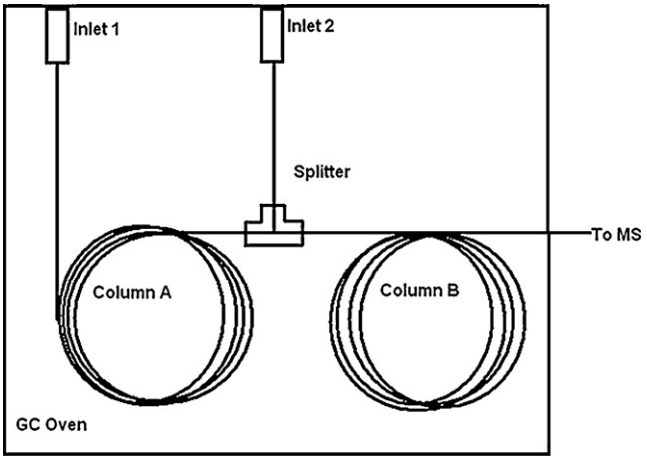

Fig. 1. Schematic of backflushing setup.

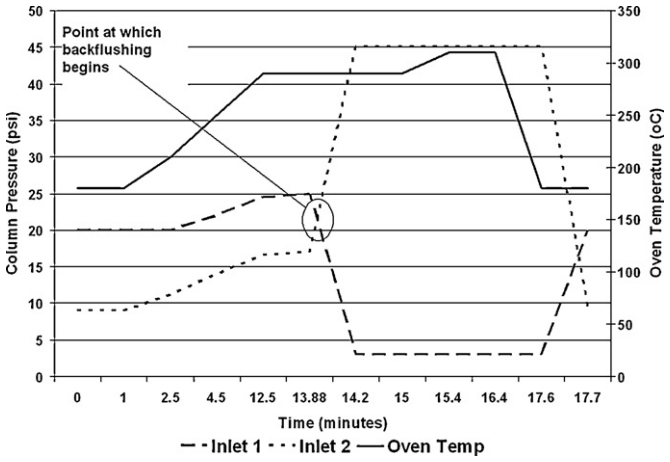

Fig. 2. Inlet pressures and oven temperature program of backflushing method.

systems. At present the use of such systems appears to be relatively limited [9–11], a review of the literature regarding doping control samples revealed no reported methods using a backflushing system. In this communication we describe the novel use of a simple, low cost backflushing system to produce significant benefits in terms of throughput and system robustness. We believe that this is the first reported use of such a low cost system to facilitate column backflushing and one that could be reproduced in any GC system that has a spare electronic flow controller.

2. Experimental

A schematic of the backflushing setup employed is shown in Fig. 1. An extremely low dead volume, inert glass lined tubing (GLT^{TM}) T-piece (SGE Analytical Science, Victoria, Australia, PN 123710) was installed between two 15 m ZB5-MSi (0.25 mm × 0.25 μm) analytical columns (Phenomenex, Macclesfield, UK, PN 7EG-G018-11). The GC–MSMS system used was a Varian 3800 GC equipped with twin injectors and electronic flow controllers coupled to a Varian 1200L triple quadrupole mass spectrometer (Varian, Oxford, UK). The inlet end of column A (front column) was inserted into a Varian 1177 split/splitless injector (inlet 1) as per the manufacturers instructions. The detector end of column A was installed into the T-piece using SilTite^{TM} ferrules. Column B (rear column) was installed into the T-piece using SilTite^{TM} ferrules and through the transfer line of the mass spectrometer and into the ion source as per the manufacturers instructions.

A 30-cm piece of deactivated silica tubing (0.25 mm ID) was installed into the second injector (inlet 2) as per the manufacturers instructions. The other end of the deactivated silica was installed into the third port of the T-piece connector using SilTite^{TM} ferrules.

A previously developed gas chromatography method for the analysis of anabolic steroids in equine urine was used as the basis for investigations into the backflushing system. The original method comprised of a 21.50-min analytical run, including a 4-min high temperature hold. Total cycle time, including oven cool down time was 23 min. The MS source was operated in electron ionization (EI) mode at an electron energy of 70 eV. The source temperature was 220 °C and the transfer line temperature was 280 °C. Helium was used as the carrier gas and operated in the constant flow mode at a column flow of 1.1 mL/min.

Setup of the backflushing conditions was performed by the repeat injection of a mixed anabolic steroid standard containing boldenone, boldienone, clostebol, ethisterone, methandriol, nandrolone, norethandrolone, testosterone, 5α-androstane-3β,17β-diol, 5α-estrane-3β,17α-diol 5α-estrane-3β,17β-diol, 5(10)estrene-3β,17α-diol, 17α-methyl-5α-androstane-3α,17β-diol, 17α-methyl-5α-androstane-3β,17β-diol and 17α-ethyl-5β-estrane-3α-17β-diol. The mixed steroid standard was evaporated to dryness under oxygen free nitrogen before being derivatised using 30 μL of a mix of MSTFA:NH_4I:ethanethiol (10 mL:60 μL:30 mg, heated at 80 °C for 2 h). 2 μL of the derivatised sample was injected into inlet 1.

The backflushing method was designed to be used as a screening method for doping control purposes. Anabolic steroid screening methods are non-quantitative and full quantitative method validation is not required. The method was validated as per ISO/IEC 17025 guidelines and shown to be fit for purpose. The suitability of the method for the analysis of equine doping control urine samples was confirmed over the course of a 3 months evaluation study, during which all equine urine samples received at HFL Sport Science were analysed using the backflushing SRM method and the original non-backflushed SIM method.

3. Results and discussion

Initially the pressure applied to the two inlets was determined by trial and error with the steroid standard mix injected using the conditions from the previously developed method whilst the pressure on inlet 2 was varied from run to run. Over the course of a small number of injections a suitable ratio of pressure on inlet 1 against inlet 2 was determined. A difference in pressure of 8 psi between the two inlets was found to produce a chromatogram almost identical to that seen in a single 30 m column system with no T-piece installed. If too low a pressure was applied to inlet 2 then no peaks were observed, presumably due to the analytes exiting the T-piece into the short piece of deactivated silica and entering inlet 2 rather than proceeding towards the mass spectrometer. If too high a pressure was applied to inlet 2 then the retention times of the analytes were increased significantly.

Once inlet pressures had been established which reproduced the chromatogram achieved with a single 30 m column system then further injections of the steroid standard mix were used to fine tune the backflushing conditions. Over the course of a number of injections it was found that commencing backflushing at 13.8 min by decreasing the applied pressure on inlet 1 and increasing the pressure on inlet 2 allowed all analytes of interest to be detected whilst reducing late eluting compounds entering column B to a minimum. The final conditions of pressure on inlets 1 and 2 and the oven temperature program are shown in Fig. 2.

No significant differences in method performance were observed between the original single column method and the back-

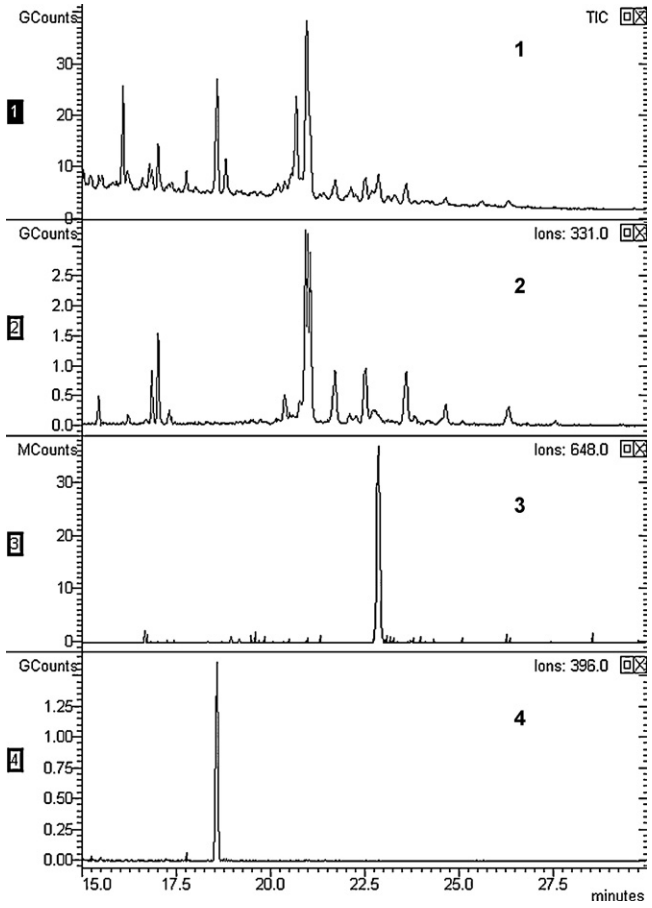

Fig. 3. Total ion current chromatograph (1) of an EI full scan (100–700 *m*/*z*) acquisition of an equine urine extract acquired using extended 30-min acquisition (15–30 min displayed). Traces 2, 3 and 4 shown extracted ion chromatograms for *m*/*z* 331, 648 and 396.

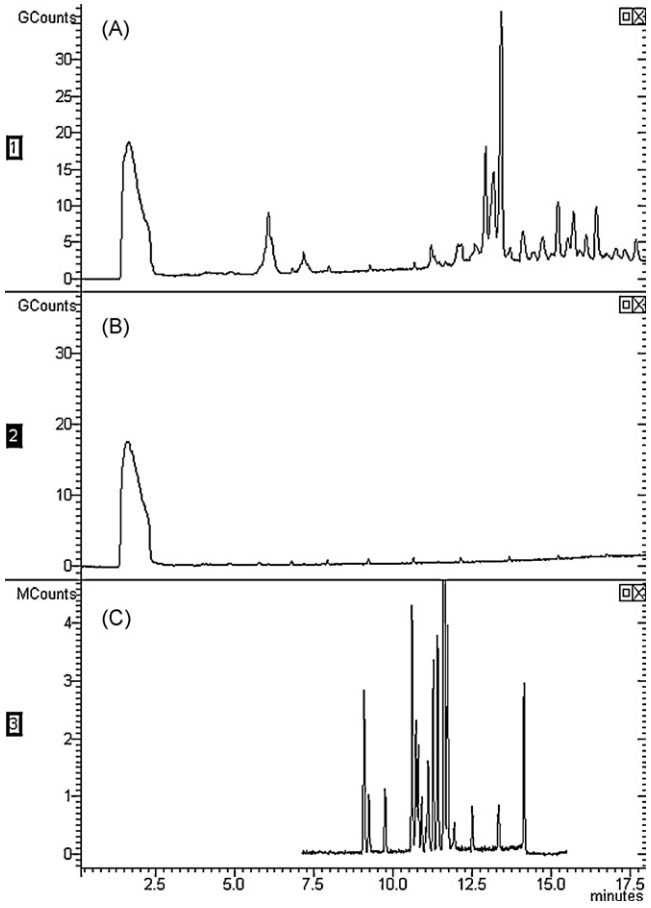

Fig. 4. Total ion current chromatograph of blank vial injection following previous injection of equine urine extract using non-backflushed method, showing carry-over of late eluting components, 0–18 min displayed (A). TIC of blank vial injection following previous injection of equine urine extract using backflushed method, showing absence of carry-over of late eluting components, 0–18 min displayed (B). TIC of anabolic steroid mixed standard to show retention times of analytes of interest, 7–15.50 min displayed (C).

flushing method. Retention times of the later eluting analytes (RT of 14 min and above) were slightly reduced using the backflushing method as the pressure on column B was increased once backflushing commenced, however, this did not adversely affect method capabilities.

To assess the potential problem presented by late eluting components in equine urine extracts an extended full scan analysis was performed. The standard 21.50-min analytical run was extended to 30 min by increasing the final hold time by 8.50 min, giving a total hold time of 12.50 min (at 310 °C). The total ion chromatogram and selected extracted ion chromatograms (*m*/*z* 331, 648 and 396) from this injection are shown in Fig. 3. This shows that in an equine urine extract there are a large number of late eluting components that would not be eluted from the capillary column using the standard method conditions. These components potentially remain in the column leading to problems with chromatography or carry-over into subsequent injections. Carry-over of these late eluting materials can significantly affect method performance, causing interference in SIM or SRM acquisitions and fouling the ion source, leading to reduced sensitivity. The selected ion chromatograms of *m*/*z* 331, 396 and 648 show that the late eluting interferences cover a wide mass range and are present in the same mass ranges as the derivatised steroids of interest.

In order to assess the potential of the backflushing method to remove these late eluting components from the capillary column the following injection sequence was performed using a full scan acquisition covering the mass range 100–700 *m*/*z*:

1. Equine urine extract injected using standard non-backflushed method (21.50 min total run time, 4 min hold at 310 °C).
2. Blank injection (empty vial) injected using extended non-backflushed method (30 min total run time, 12.50 min hold at 310 °C).
3. Equine urine extract injected using backflushing method (17.80 total run time).
4. Blank injection (empty vial) injected using extended non-backflushed method (30 min total run time, 12.50 min hold at 310 °C).

The results of the blank injections (2 and 4) are shown in Fig. 4. Also displayed is the anabolic steroid standard mix (acquired using backflushing method, 17.80 total run time, data acquisition from 7.00–15.50 min), to show the retention times of the analytes of interest.

Comparison of the two chromatograms clearly shows the advantages gained from the use of the backflushing technology. Without the use of backflushing, Fig. 4A, the subsequent blank injection contains a large amount of the late eluting components which remain on the column from the previous injection of the equine urine extract. These components are carried-over and eluted in the subsequent blank injection and appear in the chromatogram in the same region as the analytes of interest, Fig. 4C.

Table 1
Comparison of backflushed and non-backflushed methods for the detection of anabolic steroids in equine urine.

	Non-backflushed	Backflushed
Cycle time	23 min	18.2 min
Throughput improvement	n/a	+26%
Ion volume replacement	50 extracts	200 extracts
Source cleaning	3–4 weeks	3–6 months
Column trimming	3–6 months	Not required to date[a]

[a] 6 months+.

Use of the backflushing method completely eliminates the carry-over of late eluting components, Fig. 4B, which are efficiently removed from column A and exit the instrument via the split vent of injector 1. Small peaks seen in the chromatogram, Fig. 4B, were confirmed by MS to be contamination from the septa used to cap the GC vials.

The applicability of the backflushing method to routine doping control samples was demonstrated by the analysis of over 1000 equine urine extracts. Table 1 shows the advantages produced by the backflushing method in comparison to the non-backflushed method. The decision of at which point to perform instrument maintenance was based on the analysis of system suitability samples and evaluation of chromatographic performance by experienced GC–MSMS analysts. Criteria for acceptance of the system suitability standard were previously established and instrument maintenance initiated once performance dropped below these criteria. Initial instrument maintenance consisted of ion volume replacement and if this did not return performance to an acceptable level then source cleaning was performed.

The backflushing method offers a 26% improvement in sample throughput based on cycle time alone. In addition to the saving in cycle time there are also significant gains to be made in reduced instrument maintenance. Ion volume replacements were shown to be required far less frequently and source cleaning, and hence instrument down time, was also greatly reduced. Previously, trimming of the analytical column was performed on a regular basis as late eluting components built up on the column and had a detrimental effect on chromatographic performance. With the backflushing system installed column trimming is much reduced, to date following use for 6 months no column trimming has been required. This also eliminates the need to update retention times as the column is shortened. As the system uses two 15 m capillary columns it is a simple task to replace the entire column A if the need arises. Indeed, with the correct setting of pressure on inlet 2 this procedure can be performed without the need to vent the mass spectrometer.

It should be noted that the system reported here is not solely suited to triple quadrupole GC–MSMS systems. It could be equally as beneficial if installed in any system using a gas chromatograph, regardless of the detection system.

4. Conclusions

The use of backflushing technology was investigated and found to offer significant increases in sample throughput and instrument robustness. Backflushing was implemented by the use of a simple, low cost T-piece, readily available from instrument consumable suppliers and the utilization of a spare electronic pressure controller (inlet 2) already installed on the gas chromatograph. The backflush setup described in this paper would be simple to modify in order to make it applicable to any current GC–MS or GC–MSMS method, with similar advantages in cycle time and instrument robustness likely to be achieved.

Utilizing triple quadrupole GC–MSMS and the described backflush technology the throughput and turnaround times attained with three single quadrupole instruments can be obtained with two instruments. The expected improvements in coverage of analytes, sensitivity and ease of data checking have been attained along with a significant decrease in the requirement for instrument maintenance.

Acknowledgement

The authors would like to acknowledge the continued support of the British Horseracing Authority.

References

[1] M.C. Dumasia, E. Houghton, S. Sinkins, J. Chromatogr.-Biomed. Appl. 377 (1986) 23.
[2] P. Teale, E. Houghton, Biol. Mass Spectrom. 20 (1991) 109.
[3] M.H. Choi, J.Y. Kim, B.C. Chung, Anal. Lett. 32 (7) (1999) 1313.
[4] N.L. Hungerford, B. Sortais, C.G. Smart, A.R. McKinney, D.D. Ridley, A.M. Stenhouse, C.J. Suann, K.J. Munn, M.N. Sillence, M.D. McLeod, J. Steroid Biochem. Mol. Biol. 96 (3–4) (2005) 317.
[5] F. Guan, C.E. Uboh, L.R. Soma, Y. Luo, J. Rudy, T. Tobin, J. Chromatogr. B 829 (1–2) (2005) 56.
[6] E.N.M. Ho, D.K.K. Leung, T.S.M. Wan, N.H. Yu, J. Chromatogr. A 1120 (1–2) (2006) 38.
[7] Y. You, C.E. Uboh, L.R. Soma, F. Guan, X. Li, J.A. Rudy, Y. Liu, J. Chen, Rapid Commun. Mass Spectrom. 23 (13) (2009) 2035.
[8] M.S. Klee, J. Sep. Sci. 32 (2009) 88.
[9] K.L. Perry, J. Chromatogr. Sci. 28 (12) (1990) 624.
[10] P.L. Wylie, C. Tu, LC–GC North Am. 25 (6 Suppl.) (2007) 40.
[11] M.J. Gómez, M.M. Gómez-Ramos, A. Agüera, M. Mezcua, S. Herrera, A.R. Fernández-Alba, J. Chromatogr. A 1216 (18) (2009) 4071.