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Increased Speed of Steroid Analysis by Capillary GC and GC-MS; Effects of Sample Pretreatment and Sample Introduction

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Sample introduction
Separation speed

Summary

Quantitative analysis of steroids and their metabolites in urine samples calls for increased speed of sample clean-up, of the derivatization procedure, and of separation. A fast procedure for sample pretreatment, which can be performed within 8 hours, is introduced and evaluated. It is shown that use of fast pretreatment in combination with narrow bore columns, which are compatible with existing instrumentation, can considerably increase laboratory throughput. The effect of different sample introduction techniques (e.g. splitless, on-column, and moving needle) on column efficiency and resolution is demonstrated and discussed.

1 Introduction

Steroid profiling and quantitative steroid analysis from urine samples is widely used in health institutions and *inter alia* by sports organizations for detection of abuse of the forbidden anabolic steroids by athletes. In the literature many procedures can be found dealing with the pretreatment of urine prior to GC or GC-MS analysis. In most cases sample pretreatment takes a few days, and GC analysis 1 to 1.5 hours.

During the past few years many researchers in the field of steroid analysis have focused their attention on speeding up the sample pretreatment procedure. The extraction of steroids and steroid conjugates of urine by "Sep-pak" C-18 cartridges instead of XAD-2, has been rapidly accepted since its introduction by *Shackleton and Whitney* [1] and *Heikinen et al.* [2], as a convenient, fast, and reliable method. Enzymatic hydrolysis and solvolysis largely deter-

mine the overall time for pretreatment of the sample. A fast procedure for both hydrolysis and solvolysis of steroid conjugates subjected to a preliminary group separation was introduced by *Axelsson et al.* [3]. These workers reported good results.

With the current state of the art in column technology it is possible to produce highly deactivated and thermostable columns. Derivatization of groups of steroid (e.g. androgens, estrogens, anabolics) no longer appears to be a must in GC analysis. *Kovarich and Munari* [4] reported the separation of underivatized steroids on persilanized columns. Use of TMS or MO-Me derivatives, instead of the widely applied MO-TMS derivatives, will decrease sample preparation time.

Theoretically, reduction of the column inner diameter is an obvious route towards shorter analysis times in capillary gas chromatography. The practical feasibility of this approach was convincingly demonstrated by *Desty et al.* [5] as long ago as 1962. Since then this approach received little attention until *Schutjes et al.* [6] recently showed that high speed analysis can be applied to all kinds of samples with highly deactivated narrow bore capillary columns. In this paper we concentrate on the practical problems which need to be overcome if fast steroid analysis is to be performed. Both the sample pretreatment as well as the GC analysis using different injection techniques will be discussed. The twin-track approach of speeding up the sample pretreatment in steroid analysis while using fast GC analysis in narrow bore capillary columns leads to tremendous time saving, so that the laboratory throughput can be increased considerably.

Dedicated to Denis Desty on his 60th birthday.

2 Experimental

2.1 Materials

All solvents were analytical grade. Hexane and ethyl acetate were redistilled in all-glass equipment prior to use. "Helix pomatia" intestinal juice was obtained from Pharma Industry (Clichy, France). Methoxyamine hydrochloride was supplied by Applied Science Laboratories (State College, PA, USA) and the derivatization reagents are supplied by Pierce (Rockford, Ill, USA). Other chemicals are from Merck (Darmstadt, FRG). "Sep-pak" C-18 cartridges are obtained from Waters Associates.

2.2 Chromatography

Throughout the investigation several GC instruments were used: Perkin Elmer F30 and Sigma 2B, Hewlett Packard 5880 and 5790, all equipped with a flame ionization detector. Helium was used as the carrier gas in all cases. Except for the Perkin Elmer F30, which had a "moving needle" injector, the samples were introduced with a splitless system. For the comparative experiments using different injection techniques the Hewlett Packard 5880 was provided with a "moving needle" system according to de Jong [7] or a SGE on-column injector. Experimental conditions, column dimensions, and stationary phases are given with the legends to the figures or are mentioned in the text.

Table 1

Procedures for sample pretreatment.
A. Conventional (Leunissen) procedure.
B. Fast procedure

	A.	B.
1st day	- Extraction with "Sep-pak" - Hydrolysis with <i>Helix pomatia</i> Overnight 18h/37°C	- Extraction with "Sep-pak" - Hydrolysis with <i>Helix pomatia</i> 1h/60°C - Solvolysis with HCl 1h/50°C - Methoximation 1h/60°C - Trimethylsilylation 2h/110°C - GC or GC-MS analysis
2nd day	- Solvolysis with HCL overnight 18h/45°C	
3rd day	- Methoximation 1h/60°C - Trimethylsilylation overnight 18h/80°C	
4th day	- GC or GC-MS analysis	

2.3 Procedures for Sample Pretreatment.

Two methods of sample pretreatment are given in conjunction with the time required for the different steps in Table 1. The first procedure is almost identical to that developed in our laboratory by Leunissen [8] in 1979. In the original procedure the extraction of steroids and steroid conjugates from the urine samples was performed using "Amberlite XAD-2". Schackleton and Whitney [1] indicated that XAD-2 was much less efficient (about 30%) than "Sep-pak". This is in agreement with our own findings ("Sep-pak" 95% recovery, XAD-2 70%). Leunissen reported an overall recovery for XAD-2 of 95% for urine samples of subjects to whom ^3H -cortisol had been administered. These discrepancies are most probably caused by batch-to-batch differences of XAD-2. Therefore we decided to use "Sep-pak" C-18 cartridges for the extraction and modified Leunissen's procedure at this point. The second procedure incorporates fast hydrolysis and solvolysis. The incubation temperatures are increased, permitting a much shorter incubation time as described by Axelsson *et al.* for special fractions of steroid conjugates [3]. In both procedures MO-TMS derivatives are prepared. Except for steroids having a tertiary 17 α -OH group adjacent to a C-20-OH group which need a longer reaction time, reaction is complete within 15 min at 80°C. At 110°C reaction is complete within 2 hours for all steroids. For the second (fast) procedure an overall processing time of about 8 hours can be obtained, starting with the arrival of the sample in the laboratory and ending with the reporting of the results.

3 Results and Discussion

3.1 Sample Pretreatment

In order to investigate the equality comparability of the two methods given in Table 1, urine samples of a healthy adult female were treated according to both procedures. The first procedure served for reference purposes as it had been extensively optimized by Leunissen [8] using radioactively labeled steroids. Relative peak heights were calculated with respect to $n\text{-C}_{32}$ as internal standard. Height measurements appeared more reliable than peak areas even compared to electronic integration. The recovery of the fast method relative to the slightly modified "Leunissen" method and the standard deviations for both methods are given in Table 2. Representative chromatograms are shown in Figure 1, for both procedures. This figure demonstrates that with the fast procedure the recoveries of most urinary steroids are not significantly different from those of the more time-consuming method.

Four metabolites showed significant losses. Three of these, 11 β -hydroxyandrosterone, α -THF, and α -cortol are metabolites of cortisol. An average recovery of all the cortisol metabolites of 90% was observed, whereas Axelsson

Table 2

Recovery values of the fast pretreatment procedure, using peak heights calculated relative to *n*-C₃₂ and with the conventional method as a reference.

Comp.	Standard deviation (in %)		Recovery of Procedure B (in %)
	Procedure A	Procedure B	
1 A.	3.6	3.1	95
2 E.	2.9	2.3	102
3 DHEA	4.2	3.8	106
4 11-O-A+11-O-E	3.6	1.3	100
5 11-OH-A	5.2	2.6	77
6 11-OH-E	11.2	1.5	105
7 P.D.	4.7	3.4	118
8 P. T.	8.6	2.2	98
9 E-III	10.5	6.6	103
10 THE	4.2	4.2	98
11 THA	5.1	2.4	98
12 THB	2.6	4.2	93
13 α -THB	0.8	2.5	61
14 α -THE + THF	5.8	4.3	93
15 α -THE	5.8	3.5	54
16 α -Cortolone	9.4	6.8	98
17 β -Cortolone+			
β -Cortol	12.4	11.9	103
18 α -Cortol	15.9	6.8	80
Mean values	6.47	4.08	93.7

Figure 1

Comparative chromatograms of the sample pretreatment procedures. A: procedure according to Leunissen; B: fast procedure. The numbers refer to the components mentioned in Table 2.

GC instrument: Perkin Elmer F30 equipped with a moving needle injector. Column: 30 m $\times$ 0.25 mm glass, coated with OV-1. Injection port temperature: 250°C, Detector port temperature: 300°C.

reported an average recovery of cortisol metabolites of 86% for the glucuronide fraction using fast hydrolysis. The mean overall recovery of the fast procedure is 94%. The mean standard deviations for the two procedures do not differ much and are in good agreement with the mean standard deviation reported by Leunissen: 7.8% (range 6.2–9.6%).

Except for the above mentioned losses of some cortisol metabolites, the differences with respect to efficiency and reproducibility using the "Sep-pak" cartridges and the fast procedures for hydrolysis and solvolysis are not significant compared to the conventional pretreatment.

3.2 Derivatization

As already mentioned TMS derivatives can be prepared within 2 hours at 110°C. Another way of derivatizing steroids is permethylation, a method also used for the analysis of amino acids and fatty acids. Permethylation was introduced by Corey and Chaykovski [9]. The permethylation of steroids was initially described by Thomas [10]. The steroid mixture is dissolved in 100–200 μ l dimethyl sulfoxide (DMSO) and an excess of DMSO⁻ (dimethylsulfoxide carbanion) in an ultrasonic bath. After addition of an

amount of CH₃I equal to the excess of DMSO⁻ and ultrasonic vibration for 30 minutes, the reaction is stopped by adding 0.5 ml of water. The reaction products, the permethylated steroids, were extracted with 0.5 ml CH₂Cl₂. Acceptable results were reported for mono- and dihydroxy-17-ketosteroids by Tielemans [11]. Reduction of the reaction time hardly influenced the results. For several androgens, however, more than one peak was observed (artifacts, more than one derivative for one steroid). This will result in a huge number of peaks appearing in the chromatogram and an inferior reproducibility for urine samples after extraction and hydrolysis.

Figure 2

Steroid profile of a pregnancy urine, after methoximation and methylation with methyl iodide.

GC instrument: Perkin Elmer Sigma 2B, splitless injection. Column: 25 m $\times$ 0.25 mm fused silica, coated with CP-Sil 5. Injection port temperature: 275°C. Detector port temperature: 300°C.

These results could be improved considerably by methoximation of the steroids prior to methylation as shown in **Figure 2**. From this chromatogram it can be concluded that especially the androgens and estrogens are easily derivatized. Identification was performed by GC-MS. More research has to be done in order to improve reproducibility and to define the applicability. Because of the results obtained so far for androgens, MO-Me derivatives might be an attractive alternative to MO-TMS derivatives in detection of the abuse of anabolic steroids.

The low volatility, instability at high temperatures, and sensitivity to adsorption at the GC column wall and the instrument are the main reasons for derivatization of steroids prior to GC or GC-MS analysis. Considerable improvements in the deactivation of the column wall and an increased thermostability of columns with crosslinked stationary phases have been achieved as a result of recent progress in column technology.

Particularly narrow bore columns as described by *Schutjes et al.* [12] have shown extremely good deactivation characteristics. This means that the analysis of non-derivatized steroids deserves more attention. The separation of non-derivatized urinary and of some anabolic steroids is demonstrated in **Figure 3**.

3.3 GC Analysis

The speed of the GC or GC-MS analysis becomes more important when the time for the pretreatment of the sample is greatly reduced, particularly if large numbers of samples have to be analyzed. For conventional capillary columns ($L = 25$ m; $I.D. = 0.2 - 0.3$ mm) the run time for a GC analysis is about 1 hour. Improvement of the speed of separation by shortening the column length or increasing the linear carrier gas velocity will result in a reduced separation efficiency. For splitless or on-column sample introduction and for temperature programmed operation the cooling time of the oven between subsequent injections will have a negative effect on the GC run time for one sample. Therefore the use of high-boiling solvents in combination with the above-mentioned injection techniques is preferred over the use of low-boiling solvents. *Schutjes et al.* [6] have recently shown that the speed of analysis can be considerably increased by reducing the column internal diameter.

Under experimental conditions leading to identical resolution, the analysis time is inversely proportional to the inner diameter of the column. This is true not only for isothermal operation but also for temperature-programmed conditions. The programming rate must be increased with

Figure 3

The separation of non-derivatized steroids on the 0.1 mm I.D. column (cf. Table 3).

A: urinary profile; B: some anabolic steroids. GC instrument: HP 5790, splitless injection, Operating conditions: cf. Figure 2.

Figure 5

Comparative chromatograms of splitless and on-column injection on 0.3 mm and 0.2 mm ID-columns.

For explanation, see text.

Figure 6

Comparative chromatograms of moving needle injection on the three columns mentioned in Table 3.

For explanation, see text.

Figure 4

Fast steroid profiling.

The separation of MO-TMS derivatives on an 8 m $\times$ 50 μ m column, coated with OV-101. Isothermal at 250°C, split injection.

The peak numbers refer to the components mentioned in Table 2.

decreasing column inner diameter. An illustration of a fast steroid analysis using a 50 μ l ID column with split injection and a very high split ratio is given in **Figure 4**. The separation of the whole steroid profile is complete within 11 minutes. Obviously, this speed can be further improved by using H₂ as the carrier gas instead of helium.

Considering their excellent deactivation (cf. previous section) narrow-bore columns seem to be extremely useful in fast chromatographic analysis and thus also in steroid analysis. With respect to instrumentation, however, some problems have to be solved. Reduction of the inner diameter requires a high column inlet pressure. For columns with an inner diameter smaller than 0.1 mm external pressure regulators, capable of handling increased inlet pressures up to 50 bar, have to be installed. Extremely narrow peaks are produced and thus the time constants of electrometers, integrators, strip chart recorders, etc. have to be small in order to prevent peak distortion. Modern gas chromatographs fulfil these requirements, although accuracy is limited for fast eluting peaks when using columns with an inner diameter below 0.1 mm.

3.4 Sample Introduction

In steroid analysis sample introduction is mainly performed using moving needle, splitless, or on-column injection. Both the moving needle and on-column injection are least discriminative, highly reproducible, and accurate systems. Unfortunately, automation of the moving needle is not available. The applicability of automatic on-column sample

introduction, introduced a few months ago by *Trestianu* [14], has to be evaluated in daily practice. If large amounts of samples are to be analyzed and automatic sampling is required, splitless sample introduction is favorable in this particular case.

In order to investigate the column performance in relation to the column diameter for various injection techniques, three columns with inner diameters between 0.1-0.3 mm were coupled to the above mentioned injection systems. The smallest diameter (0.1 mm) was selected because it is commercially available and can be handled without any modification of a modern GC instrument. For the column characteristics the reader is referred to **Table 3**.

Equal amounts (1 μ l) of MO-TMS derivatives of a synthetic mixture of 13 urinary steroids containing *n*-C₂₄ and *n*-C₃₂ as standards and with *n*-hexane as the solvent were introduced into the column by means of splitless, on-column, and moving needle injection systems. In all the experiments the following temperature program was used: initial temperature 60°C (for on-column 80°C), after 1 minute heated ballistically to 230°C, followed by a temperature increase to 290°C at a programming rate of 3°/min. Under these temperature conditions the high boiling compounds, and thus the steroid derivatives, are trapped in the first section of the column. The peak shapes will inform us about possible injection errors.

On-column injection was performed without using a retention gap, as suggested by *Grob* [13], to prevent excessive peak distortion. For connection of the fused silica columns with the moving needle injector, the method described by *de Jong* [7] was slightly modified. The evaporation part was exchanged with the glass liner in the injection port. Due to the difference in the phase ratios of the 3 columns (cf. Table 3) and because 3 different stationary phases were used in this study a straightforward demonstration of the effect of the column diameter on the speed of analysis is not possible.

Table 3

Compatibility of column inner diameter and some injection techniques.

Characteristics of the columns.

	Inner diameter (mm)		
	0.32	0.2	0.1
Stationary phase	CP-Sil-5-CB	OV 1	CP-Sil-8-CB
Length (m)	25	12.5	8.8
Film thickness			
(μ)	0.11	0.33	0.09
Phase ratio	672	150	267
Theoretical plates	80,000	52,500	80,000
Plate height (mm)	0.31	0.24	0.11

The chromatograms obtained after splitless and on-column injection on the 0.3 and 0.2 mm I.D. columns respectively are compared in **Figure 5**. The splitless injections, after proper optimization, do not lead to any problems and good chromatograms are obtained. Severe peak broadening is observed for on-column injection onto the 0.2 mm I.D. column. Peaks just separated after splitless injection are not separated after on-column injection. The result gives neither qualitative nor quantitative information. On-column injection on the 0.3 mm column shows better results. Although the separation efficiency is slightly decreased, neither quantitation nor identification will suffer. Use of a retention gap, if possible, is expected to improve the chromatograms of on-column injections.

The chromatograms of a moving needle injection on the 3 columns is shown in **Figure 6**. The split vent was closed during the transfer of the sample into the column. The 0.1 mm column is severely overloaded, whereas the 0.3 and 0.2 mm columns show symmetrical peak shapes. Injection at an elevated initial oven temperature of 230°C, to avoid trapping of the high-boiling compounds, does not influence the results, except for the 0.1 mm column. The transport from the needle into the column is too slow and, in addition to overloading, tailing is observed.

A proper chromatogram with the 0.1 mm column using the moving needle injection technique is obtained when a split ratio of 1–10 is established; overloading is not observed, nor is peak tailing at an initial oven temperature of 230°C, as can be seen in **Figure 7**.

Figure 7

Chromatogram of moving needle injection on the 0.1 mm I.D. column, split ratio 1:10.

The programming rate is 6°/min.

4 Conclusions

The time required for sample pretreatment in steroid profiling ("Sep-pak" extraction in combination with fast hydrolysis/solvolysis) is reduced to about 8 hours. Recoveries and reproducibility are not significantly different from those reported by *Leunissen* [8].

If large amounts of samples have to be analyzed the laboratory throughput will be limited by the GC run time. Reduction of the inner column diameter is an attractive way of increasing the speed of GC analysis without loss of separation efficiency. However, optimization of the separation efficiency becomes more critical and sample introduction more difficult.

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