Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (2)

M. Donike
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

Sport und Buch Strauß, Köln, 1995

H. Geyer, U. Mareck-Engelke, E. Nolteernsting, G. Opfermann, M. Donike:
The Matrix Problem in Calibration of the GC/MS for Endogenous Steroids. The Search for an Artificial Urine
In: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (2). Sport und Buch Strauß, Köln, (1995) 199-209

H.Geyer, U.Mareck-Engelke, E.Nolteernsting, G.Opfermann and M.Donike

The Matrix Problem in Calibration of the GC/MS for Endogenous Steroids. The Search for an Artificial Urine

Institut für Biochemie, Deutsche Sporthochschule, Köln, Germany

Introduction

The calibration of the GC/MS for determining endogenous steroids with pure standards is often connected with problems. 1). The response for known amount of steroids is higher in the urine matrix than in a pure standard mixture. 2). The area ratios of steroids maybe different in urine and a pure standard mixture. 3). The calibration curves for steroids in pure standard solutions are not linear (1, 2).
These problems probably result from a missing biological matrix in solutions of pure standards and maybe connected with the injection port, the column, the ion source or the mass specific detector.

Experimental

Calibration standard

For the calibration of endogenous steroids 20 µl of a standard mixture in methanol (tab.1) is evaporated and dissolved in 100 µl of derivatisation reagent.

Internal standards

To urine samples and to the calibration standard are added 20 µl of a mixture of steroids as internal standards (tab. 2).
The methanolic solutions are evaporated to dryness under vacuo and derivatised with 100 µl of MSTFA/NH4I/ethanthiol (1000:2:3 (v:w:v) and heated for 15 min at 60°C . 3 µl of the solution are injected into the GC/MS.

Tab. 1: Standard mixture for the calibration of endogenous steroids.

Substance	Abbreviation	Concentration in methanol [ppm]
androsterone	AND	200
etiocholanolone	ETIO	200
epitestosterone	EPI	4
testosterone	TEST	4
11ß-OH-androsterone		40
11ß-OH-etiocholanolone		20
5α-androstane-3α,17ß-diol		8
5ß-androstane-3α,17ß-diol		18
dehydroepiandrosterone		40
5ß-pregnane-3α,20α-diol		100
tetraydrocortisol		100

Tab. 2: Mixture of internal standards.

Substance	Abbreviation	Concentration in methanol [ppm]
17α-methyltestosterone	MTEST	50
[2,2,4,4-$^2H_4$]-etiocholanolone	D4ETIO	50
[16,16,17,-$^2H_3$]-testosterone	D3TEST	2
[2,2,4,4-$^2H_4$]-11ß-hydroxyandrosterone	D411OH	14

Sample preparation (3)

2 ml of urine and 20 µl of an internal standard mixture (see above) are added to a Amberlite XAD-2 column. The column (pasteur pipette, closed with glass pearl, bed height 2 cm) is washed with 2 ml of bidistilled water and the absorbed fraction is eluted with 2 ml of methanol. The methanolic eluate is evaporated to dryness and the residue is dissolved in 1 ml of 0.2 M sodium phosphate buffer pH 7.

To the buffer solution, 50 µl of beta-glucuronidase from E.coli is added and hydrolysis is performed for 1 h at 50° C. The buffered solution is alkalized with 250 µl of 7% potassium carbonate solution to pH 9-10 and the steroids are extracted with 5 ml of diethyl ether or tert.-butylmethyl ether on a mechanical shaker for 5 minutes. After centrifugation the etheral layer is transferred and evaporated to dryness under vacuo.

The derivatisation is performed as described above.

GC/MS parameters
GC/MS: HP 5890/ HP 5971A (Hewlett Packard)
column: HP Ultra I (OV-1), 17m , 0.2 mm i.d., 0.11 µm film thickness
carrier gas: 1 ml helium at 180° C, split 1:10
temperature program: 180° C, 3° C per min, 229° C, 40° C per min , 320° C

1-(N,N-diisopropylamine)-alkanes (Dipas)
The Dipas used in this study were synthesized by E. Nolteernsting (4). The structure of the Dipa with a chain length of 12 carbon atoms is presented as an example in figure 1 .

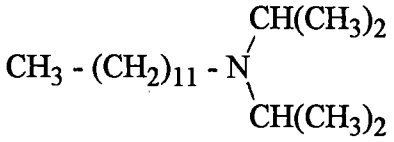

Fig. 1: Structure of 1-(N,N-diisopropylamine)-dodecane (Dipa 12).

Results and Discussion

Injection of internal standards with and without urine matrix
The analysis of the deuterated steroids of our internal standard mixture (see tab. 2) a) in the calibration standard and b) in an urine sample led to higher responses for the internal standard steroids in the urine matrix (fig. 2). Also the ratios of the peak areas of the internal standard steroids in the pure calibration mixture differed strongly from the ratios in a urine sample (tab. 3).
The increase of the detector response seems to be related to 2 factors: 3-keto-4-ene structure and concentration as can be concluded from the comparison D3TEST and MTEST in urine and pure standard (fig. 2). A similar conclusion can be deduced from the obtained peak area ratios presented in table 3.

We observe these effects on all our gas-chromatographs with mass specific detectors (all from Hewlett Packard). The extent of these effects change from day to day and from GC/MSD to GC/MSD.

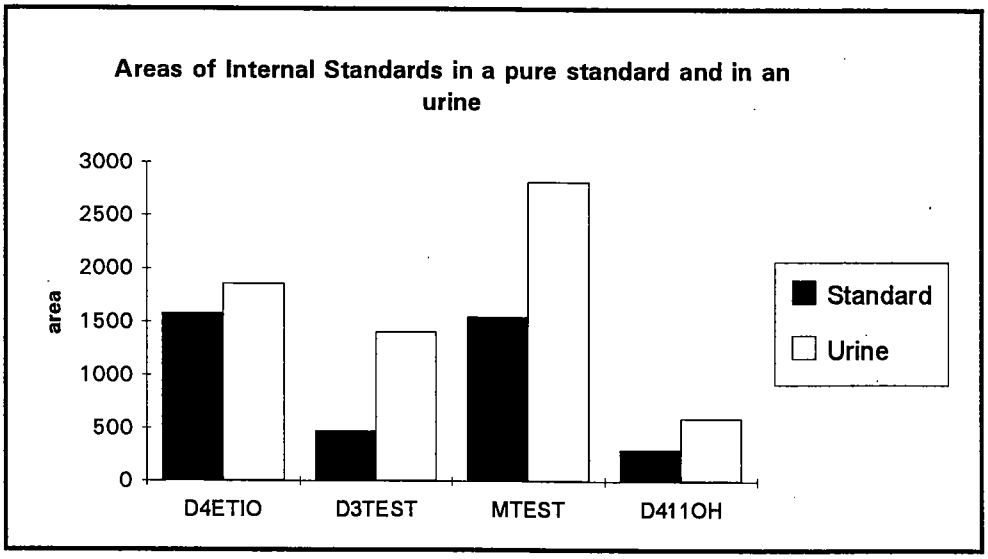

Fig. 2: Areas of the deuterated steroids of the internal standard included in a calibration standard and in an urine. The areas of D3TEST are multiplied with 10.

Tab. 3: Ratios of the areas of the deuterated steroids of the internal standard included in a calibration standard and in an urine

	D4ETIO/ D3TEST	D4ETIO/ MTEST	MTEST/ D3TEST	D4ETIO/ D411OH
Standard	3.30	1.02	3.24	5.19
Urine	1.32	0.66	2.01	3.10

Effect of different amounts of the urinary matrix

Different volumes of a normal male urine were spiked with the same amount of internal standard mixture (see tab. 2) and prepared according to the procedure described in the experimental section.

The results in table 4 show that the peak areas correlate with the urine volume processed and by this with the biological matrix injected. Also the ratios of the peak areas of D4ETIO/MTEST changed with the urine volume, respectively the biological matrix.

This study shows, that the addition of calibration standards to child urine, as it is proposed by *Linnet* (1), or to urine of postmenopausal woman is not the optimal solution to overcome the problems of a missing biological matrix as the calibration factors of the steroids change with the concentration of the biological matrix.

Another disadvantage of urine from children or postmenopausal women is the fact that these urines contain the steroids which are in the calibration standard. Therefore, a correct quantitation of the steroids of the calibration standard is difficult.

Tab. 4: Peak areas and ratios of constant amounts of internal standard steroids in different volumes of urine

urine volume [ml]	D4ETIO m/z 438 (area)	MTEST m/z 446 (area)	D4ETIO/ MTEST (ratio)
0.5	4101	7132	0.58
1.0	4805	8857	0.54
2.0	4807	9288	0.52
4.0	5604	10506	0.53
8.0	6285	12448	0.50

Calibration curve of standards testosterone/epitestosterone 6:1 (T/E 6:1) under different conditions

A calibration curve of testosterone and epitestosterone at a 6:1 ratio was prepared. The testosterone amounts ranged from 10 to 2560 ng in the sample. The standards were analyzed in different ways:

pure:as pure standards (methanolic solutions evaporated and derivatised)

child: added to a child's urine (child urine with very low amounts of testosterone and epitestosterone spiked with methanolic T/E 6:1 standards and prepared according to method mentioned above)

bio: as pure standards, but injected after 2 injections of normal urine samples

dipa: as pure standards + Dipa 14-23 10000 ng each (methanolic solutions evaporated and derivatised)

Table 5 shows the calibration factors of testosterone relative to the constant internal standard D4ETIO. A linear calibration curve for testosterone would result in constant calibration factors for each testosterone concentration. In contrary to this, all calibration factors of the different injection modes increased with the testosterone concentration. The strongest variation could be observed after injection of pure standards, whereas the addition of child urine or Dipa or the injection between two biological samples resulted in a smaller increase of the calibration factors, i.e. in a more linear calibration curve.

As shown in table 6 a strong variation of the T/E ratio (from 3.2 to 8.3) could be observed after injection of pure standard solutions. This variations of the T/E ratios decreased after the

addition of child urine (from 6.1 to 8.0) or Dipa (from 4.8 to 7.2) or the injection between bological samples (from 5.1 to 7.3).
The "memory effect" after injections of biological samples influences in an increasing sense the response of pure standards. This effect decreases with the number of injections of non-biological samples.

Tab. 5: Calibration factors of testosterone at different amounts relative to constant amounts of the internal standard D4ETIO. Explanation of "pure, child, bio, dipa" see text above.

TEST [ng]	pure	child	bio	dipa
10	1.00		1.47	1.24
20	0.90	1.36	1.46	1.20
40	1.08	1.43	1.42	1.30
80	1.06		1.59	1.57
160	1.27	1.76	1.90	1.64
320	1.61	1.73	1.80	1.92
640	1.74	1.86	2.04	1.98
1280	2.33		2.11	2.19
2560	2.41		2.08	2.19

Tab. 6: T/E ratios from peak areas of standards T/E 6:1 at different concentrations and injection under different conditions. Explanation of "pure, child, bio, dipa" see text above

TEST [ng]	pure	child	bio	dipa
10	3.2		5.5	4.8
20	3.9	6.1	5.1	4.8
40	4.8	6.4	6.2	6.2
80	5.9		6.5	6.5
160	6.9	7.2	6.9	6.9
320	7.7	7.8	7.3	7.2
640	8.3	8.0	7.1	7.2
1280	8.2		7.2	6.9
2560	7.9		7.0	6.7

Effects of Dipas

To the mixture of internal standards (see tab.2) in increasing amounts we added a solution of Dipas 14-23, 10000 ng each (methanolic solution, evaporated and derivatised). The results are presented in table 7 and 8.

The areas of D4ETIO were about 2 fold higher with Dipas than without Dipas. For D3TEST we found 2.5- till 3.8-fold higher values with Dipas (tab. 7).

The ratios D4ETIO/D3TEST and D4ETIO/MTEST decreased with increasing concentrations without Dipas. With Dipas this decrease was weaker or the ratios remained constant (tab. 8).

Tab. 7: Areas of steroids of the internal standard injected a) without and b) with Dipas 14-23, 10000 ng each. 0.25 x ISTD means that only a quarter of the normal amount of the steroids of the internal standard (see experimental part) was injected.

ISTD	D4ETIO m/z 438 (areas)		D3TEST m/z 435 (areas)	
	a)	b)	a)	b)
0.25 x ISTD	26.6	50.3	1.0	3.8
0.5 x ISTD	60.6	117.1	2.7	9.1
1.0 x ISTD	145.1	256.9	6.8	19.8
2.0 x ISTD	434.4	567.8	21.9	45.4
4.0 x ISTD	730.3	1255.2	42.4	101.7

Tab. 8: Ratios of areas of steroids of the internal standard injected a) without and b) with Dipas 14-23, 10000 ng each. 0.25 x ISTD means that only a quarter of the normal amount of the steroids of the internal standard (see experimental part) was injected.

ISTD	D4ETIO/D3TEST (ratios)		D4ETIO/MTEST (ratios)	
	a)	b)	a)	b)
0.25 x ISTD	26.31	13.20	0.76	0.65
0.5 x ISTD	22.60	12.91	0.79	0.59
1.0 x ISTD	21.34	12.96	0.74	0.59
2.0 x ISTD	19.81	12.52	0.64	0.58
4.0 x ISTD	17.24	12.34	0.60	0.60

Effect of different amounts of Dipas

To a mixture of internal standards (see tab. 2) we added different amounts of Dipas 14-23 (methanolic solutions, evaporated and derivatised).

The results in table 9 show an increase in the peak areas with increasing amounts of Dipas. The highest Dipa amount means a total Dipa amount of 200 µg.

Tab. 9: Areas of constant amounts of D4ETIO and D3TEST after addition of different amounts of Dipas 14-23. The Dipa amount means the amount of each single Dipa in the Dipa mixture.

amount of each Dipa [ng]	D4ETIO m/z 438 (area)	D3TEST m/z 435 (area)
0	175.3	17.1
250	187.2	17.1
500	193.6	17.6
1000	200.2	18.1
2500	205.5	19.2
5000	215.3	21.1
10000	225.2	24.4
20000	227.7	26.7

Effect of order of elution of Dipas

To a mixture of internal standards (see tab. 2) we added different solutions of Dipas.

- Dipa 5-14 (total amount 100 µg): Dipas are eluted before steroids of interest
- Dipa 14-23 (total amount 100 µg): Dipas are eluted within the elution range of the steroids of interest
- Dipa 22-23 (total amount 100 µg): Dipas are eluted after steroids of interest

The areas of the steroids of the internal standard are presented in table 10. The lowest peak areas are reached without Dipas or when the Dipas were eluted after the steroids of interest. If the Dipas were eluted before the steroids of interest higher areas were recorded. The best results were obtained with Dipas which were eluted over the whole elution range of the steroids of interest.

Tab. 10: Areas of steroids of the internal standard without Dipas and spiked with different Dipa solutions

- Dipa 5-14 (total amount 100 µg): Dipas are eluted before steroids of interest
- Dipa 14-23 (total amount 100 µg): Dipas are eluted over the whole elution range of the steroids of interest
- Dipa 22-23 (total amount 100 µg): Dipas are eluted after steroids of interest

Dipa	D4ETIO m/z 438 (area)	D3TEST m/z 435 (area)	D411OH m/z 526 (area)	MTEST m/z 446 (area)
without	1028	66	254	1642
5-14	1805	128	464	3130
14-23	2138	175	548	3759
22-23	947	62	221	1514

Compairison of Dipa, paraffinum and n-alkanes

To the calibration standard of endogenous steroids (see tab. 1) we added Dipas, n-alkanes and Paraffinum. The results are presented in table 11. The highest peak areas for the steroids are achieved with Dipas and Paraffinum. Like the Dipas 14-23, Paraffinum is eluted over the whole elution range of the steroids of interest and leads to an increase of the peak areas.

Tab. 11: Peak areas of 3 steroids of the calibration standard (see tab.1) after addition of different substances.

- Dipa = Dipa 14-23 10000 ng each (evaporated and derivatised)
- C12 = Duodecan 1 µl (added to the derivatised standard)
- C16 = Hexadecan 1 µl (added to the derivatised standard)
- C20 = Eicosan 1 mg (added to the derivatised standard)
- Para = Paraffinum 1µl (added to the derivatised standard)

Addition	MTEST m/z 446 (area)	TEST m/z 432 (area)	EPI m/z 432 (area)
no addition	392.8	294.8	41.7
Dipa	467.2	360.1	52.0
C 12	400.0	289.2	37.7
C 16	412.0	302.9	38.4
C 20	459.6	351.4	49.2
paraffinum	480.1	372.6	55.3

Conclusion

The presented results demonstrate the extent of importance of the biological matrix in the gas-chromatographic/mass-spectrometric quantitation of urinary steroids and the steroid profile. The matrix effects can be observed on all our GC/MSD's (Hewlett Packard). The extent of the observed effects change from day to day and from GC/MSD to GC/MSD, which may be also a consequence of a certain non-constant "memory effect".

For these reasons, calibration standards should contain a similar biological matrix as urine. There are different possibilities to overcome this problem:

- Use of urine of children or postmenopausal women (especially for the determination of TEST and EPI) to prepare the calibration solution.
- Use of the "memory effect", i.e. the injection of a calibration standard after injections of urine samples
- Use of "artifical urine", i.e. the addition of Dipas or paraffinum to the calibration standards

The most convenient way would be the use of an "artifical urine" where the imitated biological matrix is available in unlimited and chemically defined quantities. So there are no problems with changes in the biological material and with the stability of biological samples. An "artifical urine" is easy to prepare and easy to add to a calibration standard. Furthermore "artificial urines" don't contain endogenous steroids as it is still the case with urines of children and postmenopausal women.
Prerequesit for the effectiveness of an "artificial" urine is that its substances are eluted over the elution range of the steroids of interest.

In our laboratory we prefer the use of solutions of Dipas 14-23 containing 10000 ng of each Dipa. The correctness of the calibration factors in the calibration standard with Dipas is controled by the comparison of the ratios of the four steroids of our internal standard in the calibration standard and in every urine sample. The possibility to use paraffinum is under investigation.

References

1. *Linnet, K.:* Effect of urine matrix on the testosterone/epitestosterone calibration curve. In: *M. Donike, H. Geyer, A. Gotzmann, S. Rauth* (eds.) 10th Cologne Workshop on Dope Analysis: 7th to 12th june 1192. Sport und Buch Strauß, Köln 1993

2. *Vobejda, C.:* Zur Frage der Linearität von Eichkurven für gaschromatographisch-massenspektrometrische Untersuchungen. Diplomarbeit. Deutsche Sporthochschule Köln, 1990.

3. *Donike, M.; Geyer, H.; Gotzmann, A.; Kraft, M.; Mandel, F.; Nolteernsting, E.; Opfermann, G.; Sigmund, G.; Schänzer, W.; Zimmermann, J.*: Dope analysis In: *P. Bellotti, G. Benzi, A. Ljungqvist* (eds.) Official Proceedings International Athletic Foundation World Symposium on Doping in Sport. IAAF, Florenz 1987.

4. *Nolteernsting, E.:* 1-(N,N-Diisopropylamino)-alkane. Ein neues Referenzsystem für gas-chromatographische und gas-chromatographisch/massenspektrometrische Untersuchungen. Examensarbeit. Deutsche Sporthochschule Köln, 1984.