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Comparison of normal-phase and reverse-phase HPLC clean up for $^{13}C/^{12}C$ analysis of endogenous urinary steroids

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Introduction

Due to the current shortage of acetonitrile the development of acetonitrile-saving methods in doping analysis is advisable. During sample preparation for $^{13}C/^{12}C$ analysis of endogenous urinary steroids by GC-C-IRMS mainly the HPLC clean up consumes acetonitrile. This is performed on a reversed phase column (RP). Acetonitrile constitutes the major part of the mobile phase. Therefore the HPLC method was migrated to a normal-phase (NP) column. n-Hexane and isopropanol make up the mobile phase. The method was originally developed for the clean up of norandrosterone samples [1]. The aims of this study were to demonstrate sufficient agreement of both HPLC methods and to validate the NP method. None of the tested steroids failed.

Materials and Methods

Validity of the NP-HPLC method was studied by the approach of linear mixing models. 6 blank urines were prepared in duplicate, whereas five of them were spiked with androsterone (A), etiocholanolone (E), pregnanediol (PD) and 11ß-hydroxyandrosterone (OHA). Table 1 lists the endogenous steroid concentration for the blank urine and the concentration of each spiked steroid.

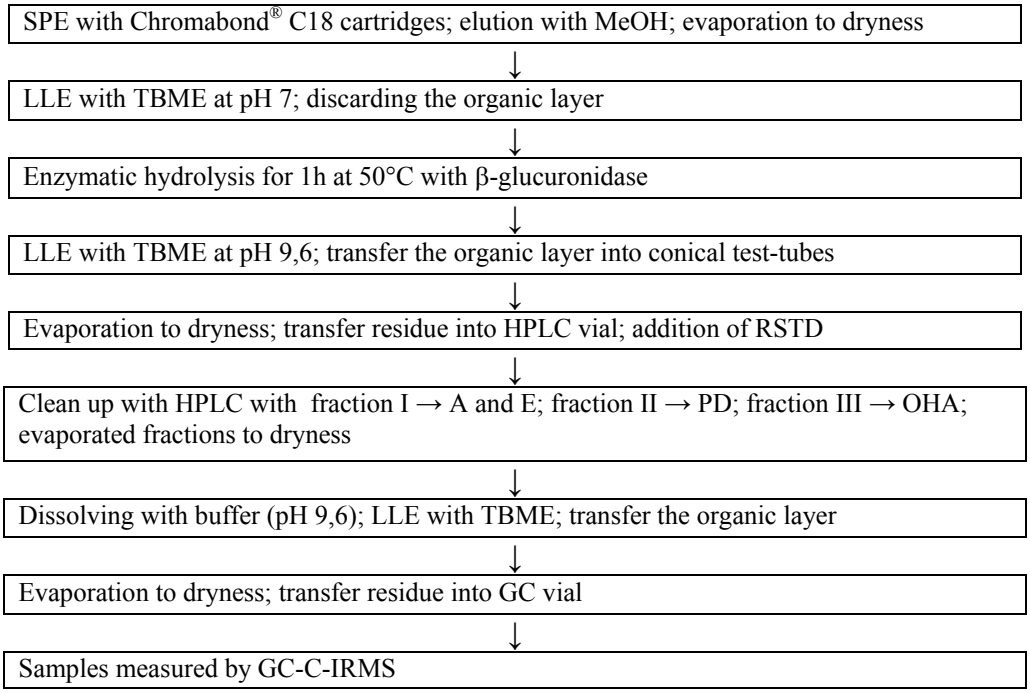
Table 1: Endogenous steroid concentrations of blank urine and amounts of the spiked steroids androsterone (A), etiocholanolone (E), pregnanediol (PD) and 11ß-hydroxyandrosterone (OHA).

Steroid	Endogenous [ng/ml]	S1 [ng]	S2 [ng]	S3 [ng]	S4 [ng]	S5 [ng]
A	1145	340	690	1150	1720	2290
E	1048	310	630	1050	1570	2100
PD	123	37	74	120	180	250
OHA	291	87	170	290	440	580

For the comparison of the methods 33 urine samples were prepared in duplicate. All samples were measured by GC-C-IRMS [3]. One sample was prepared by the NP-HPLC method and the other one by the RP-HPLC method. The results were visually compared with BLAND/ALTMAN-plots [2]. Reference limits were estimated by addition of the threefold standard deviation to the mean differences of $\Delta^{13}C$ values.

Sample preparation

Figure 1: Flow scheme of sample preparation.



Results and Discussion

Validation

As above mentioned the validation of the NP-HPLC method was carried out by means of linear mixing models.

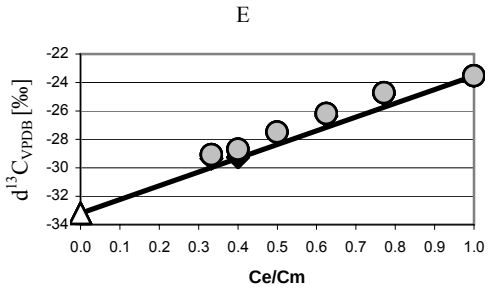


Figure 2: Linear mixing model for E. The triangle represents the $\delta^{13}C$ values of the standard, the circles the first preparation and the diamonds the second sample preparation. The solid line represents the best fit for the true concentrations.

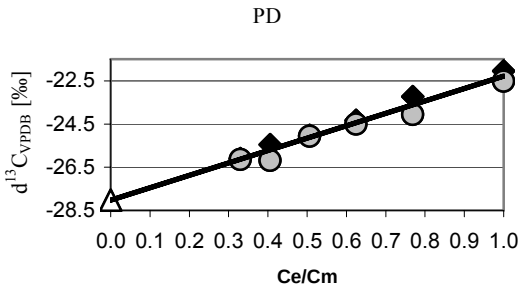


Figure 3: Linear mixing model for PD. The triangle represents the $\delta^{13}C$ values of the standard, the circles the first preparation and the diamonds the second sample preparation. The solid line represents the best fit.

The shift in $\delta^{13}C$ values of all spiked samples illustrated in Figure 2 is due to an increased recovery of endogenous E on the NP column. Due to the primarily underestimated

concentration for the endogenous steroid the $\delta^{13}C$ values have shifted to less depleted values. Figure 3 illustrates the results obtained for PD. The graphically evaluated $\delta^{13}C$ value (-28.0‰) for the standard is represented by the intercept of the line of best fit with the y-axis. This is in perfect agreement with the measured value of -28.0‰. No bias in $\delta^{13}C$ values could be observed. OHA and A were validated by means of linear mixing models too. None of the tested steroids failed. The calculated values for all linear mixing models are summarized in Table 2.

Table 2: Evaluated values for all linear mixing models referring to the equation y = a * x + b. With a representing the Δ values of the endogenous steroid and the spiked standard, b the $\delta^{13}C$ value of the standard. All values in $\delta^{13}C_{VPDB}$ [‰].

Steroid	a [‰]	SD (a) [‰]	b [‰]	SD (b) [‰]
A	7.8	0.25	-30.5	0.16
E	10.0	0.30	-33.3	0.20
PD	5.8	0.28	-28.0	0.17
OHA	-8.0	0.32	-14.6	0.18

BLAND/ALTMAN-plot

Both HPLC methods were compared by BLAND/ALTMAN-plots as illustrated in Figure 4 for $\Delta^{13}C$ of PD-A and Figure 5 for $\Delta^{13}C$ of OHA-A. In both diagrams the averages of the $\Delta^{13}C$ values are plotted against their corresponding differences. No significant systematic differences between the methods can be recognized.

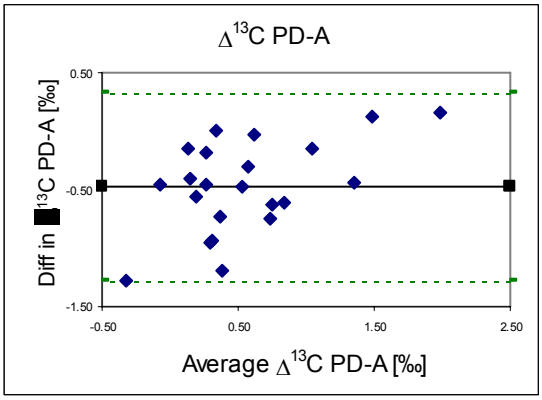


Figure 4: BLAND/ALTMAN-plot for $\Delta^{13}C$ of PD-A. The diamonds represents the mean $\Delta^{13}C$ values (x-axis) and the corresponding differences of the $\Delta^{13}C$ values (y-axis) for both HPLC methods. The dotted lines represent the twofold standard deviations. The solid blank line represents the mean difference of all samples.

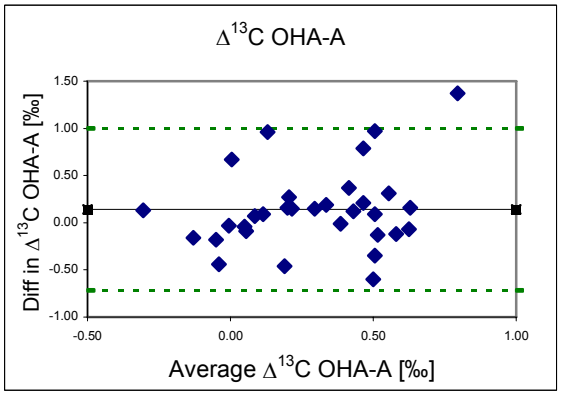


Figure 5: BLAND/ALTMAN-plot for $\Delta^{13}C$ of OHA-A. The diamonds represents the mean $\Delta^{13}C$ values (x-axis) and the corresponding differences of the $\Delta^{13}C$ values (y-axis) for both HPLC methods. The dotted lines represent the twofold standard deviations. The solid blank line represents the mean difference of all samples.

Both HPLC-methods show sufficient agreement. In contrast to PD (0.40‰ for Δ NP - RP) and E (0.29‰ for Δ NP - RP) the mean $\delta^{13}C$-values for A (-0.06‰ for Δ NP - RP) and OHA (-0.08 ‰ for Δ NP - RP) exhibit smaller differences.

Reference limits

Compared to the RP-HPLC method the $\delta^{13}C$-values of PD and E were found to be enriched after NP-HPLC clean up. This can be compensated for by appropriate adjustment of the reference limits of the corresponding $\Delta^{13}C$ values which were listed in Table 3.

Table 3: Reference limits (Ref.-lim.) for $\Delta^{13}C$ values after NP-HPLC and RP-HPLC clean up. All values in $\delta^{13}C_{VPDB}$ [‰]

	Δ (PD-E) [‰] Ref.-lim.	Δ (OHA-E) [‰] Ref.-lim.	Δ (OHA-A) [‰] Ref.-lim.	Δ (PD-A) [‰] Ref.-lim.
NP-HPLC	3.1	2.8	1.5	2.3
RP-HPLC	2.6	3.3	1.7	0.9

This results in a greater reference limit for PD-E and PD-A after NP-HPLC, whereas for OHA-E it is smaller and for OHA-A it remains approximately equal. The shift in the reference limit for PD-A was found greater than for PD-E after NP-HPLC. The reason for this is the different influence of $\delta^{13}C$ values for A and E. The $\delta^{13}C$ values for A remain approximately equal whereas the $\delta^{13}C$-values for E were enriched. Combined with enriched $\delta^{13}C$-values for PD the shift in reference limits for PD-E is smaller than for PD-A.

Conclusion

The BLAND/ALTMAN-plots suggest sufficient agreement of both HPLC methods. The NP-HPLC method was validated by means of liner mixing models. None of the tested steroids failed. Replacement of the RP-HPLC method by the NP-HPLC method is possible during screening and has already been implemented. For confirmation purpose the existing method should not be changed as this would be contradictory to the concept of population based reference limits.

References

[1] Hebestreit M, Flenker U, Fusshöller G, Geyer H, Güntner U, Mareck U, Piper T, Thevis M, Ayotte C, Schänzer W. (2006) Determination of the origin of urinary norandrosterone traces by gas chromatography combustion isotope ratio mass spectrometry. *Analyst* **131**, 1021–1026.

[2] Bland JM, Altman DG (1986) Statistical methods for assessing agreement between two methods of clinical measurement. *The Lancet* **8476**, 307–310.

[3] Piper T, Mareck U, Geyer H, Flenker U, Thevis M, Platen P and Schänzer W (2008) Determination of 13C/12C ratios of endogenous urinary steroids: method validation, reference population and application to doping control purposes. *Rapid Commun Mass Spectrom.* **22**, 2161–2175.