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Comparison of uncertainties: the standard deviations of the method of two calibration curves with different preparatory steps

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Introduction

Within the ALADIN project (EU project Nr. GTC1-2000-28005) the stability of 17β-methyl-5β-androst-1-ene-3α,17α-diol (epimetendiol, EMD) and of clenbuterol in urine at 4°C, -20°C and –80°C was to test for at least 2 years. The concentrations were determined by means of calibration curves with a working range from 0.5 to 5.0 ng/ml following the DIN 38 402 Part 51. Ten calibration urine samples were prepared by adding 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µl of the EMD working solution while clenbuterol was added from 10 different working solutions which were prepared by dilution of 10 different stock solutions. To investigate the influence of the different preparatory steps on the uncertainty the standard deviations of the method for EMD and clenbuterol are used.

Experimental

Preparation of the test urine samples: A commercially available standardised urine was spiked with clenbuterol and EMD at 2.5 ng/ml each and after 1, 2, 3, 4, 5, 6, 9, 12, 18 and 24 months 5 aliquots were measured for each of the three storage temperatures.

Preparation of the clenbuterol calibrators: 10 mg of clenbuterol were weighed five times into 100 ml calibration flasks. After filling up with methanol (MeOH) and rigorously mixing 15 µl (3 ng/20 µl), 25 µl (5 ng/20 µl), 35 µl (7 ng/20 µl), 45 µl (9 ng/20 µl), and 25 µl to 50 ml (1 ng/20 µl) were diluted to 10 ml with MeOH. 10 mg of clenbuterol were weighed three times into 10 ml calibration flasks and after treatment as above 15 µl (6 ng/20 µl), 20 µl (8 ng/20 µl) and 25 µl (10 ng/20 µl) were diluted to 50 ml with MeOH. 2 ng/20 µl were prepared by dilution of 25 µl of 10 mg of clenbuterol/50 ml MeOH to 50 ml and 4 ng/20 µl by the same procedure with 10 mg of clenbuterol/25 ml MeOH. 2 ml of urine were spiked with 20 µl of the calibrator solutions each.

Preparation of the EMD calibrators: 2 ml of urine were spiked with 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µl of a methanolic solution of 100 ng of EMD/ml which was derived by dilution of 100 µl of 10 µg of EMD/ml of MeOH to 10 ml with MeOH. The latter was prepared by dilution of 100 µl of weighed 10 mg of EMD/10 ml MeOH to 10 ml with MeOH.

Sample preparation: 25 µl of the methanolic internal standard solution (clenpenterol 4 µg/ml, ISTD for clenbuterol, as well as methyltestosterone (MeT) 4 µg/ml, ISTD for EMD) and

20 µl of KOH 5 N were added to 2 ml of the urine samples. They were extracted with 6 ml of t-butyl methyl ether by shaking for 20 minutes, centrifuging and transferring the ethereal layers. They were evaporated to dryness in vacuo and then dried in a vacuum desiccator over P_4O_{10} and KOH for at least 1 h. The residues were silylated with 100 µl of MSTFA/NH_4I/ ethanethiol (1000/2/3, v/w/v) by heating for 20 min at 60°C. After cooling to ambient temperature 3 µl of the solutions were injected into the GC/HRMS system for the simultaneous determination of EMD and clenbuterol.

Gas chromatography/mass spectrometry: GC/HRMS analyses were performed using a Finnigan MAT 95 double focussing mass spectrometer interfaced with an Agilent Technologies 6890 gas chromatograph. The derivatised samples were analysed using an Agilent Ultra 1 (OV1) fused silica capillary column (17 m length, 0.2 mm i.d., 0.11 µm film thickness). Helium was used as carrier gas and the column flow was 1 ml/min at 185°C. 3 µl of sample were injected in the split mode at a split flow of 20 ml/min. The injection port and transfer line temperatures were 300°C. The oven temperature was 185°C upon injection, then heated at a rate of 5°C/min to 320°C and held at the final temperature for 3 min. The compounds were ionised via electron impact ionisation at an electron energy of 65 eV and an emission current of 1 mA. The ion source and source probe temperature were adjusted to 240°C. A mass resolution of 4000 was employed, and the mass analysis was performed in the multiple ion detection (MID) mode using an electric field scan. Calibration of the electric fields was continuously performed using perfluoronaphthalene. Group 1 starting at 3:05 min: 242.9856 (lock mass, l) 2.73 ms, 404.9760 (calibration mass, c) 2.73 ms, 7 ions with a dwell time of 34.13 ms each, 335.0695 used for quantification of clenbuterol and clenpenterol. Group 2 starting at 5:50 min: 366.9792 (l) 2.73 ms, 455.9762 (c) 2.73 ms, 11 ions with 25.94 ms each, 358.2692 used for quantification of EMD. Group 3 starting at 10:30: 504.9697 (l) 2.73 ms, 642.9601 (c) 2.73 ms, 15 ions with 25.94 ms each, 446.3036 for quantification of MeT.

Results and Discussion

Including the first analysis at the beginning of the study 11 sets of data for each substance have been prepared and measured. Outliers were removed. The characteristics of linear regression are presented in Table 1. While within each column the curves are very similar regarding the slope b the s(x0) values exhibit a big variation mainly caused by the values at month 6. The coefficient of variation drops to 25.1 % for EMD and 21.7 % for clenbuterol when the values at month 6 are considered as outliers. In any case the t-test (Table 2) confirms that the differences of s(x0) of EMD and clenbuterol are significant.

Table 1: Calibration curves y = a + bx, number of calibration points n, residual standard deviations s(y) and standard deviations of the method s(x0): left EMD, right clenbuterol

months	n	slope b	intercept a	s(y)	s(x0) [ng/ml]	n	slope b	intercept a	s(y)	s(x0) [ng/ml]
0	10	0.0088	0.0007	0.0014	0.155	9	0.0277	0.0176	0.0086	0.309
1	10	0.0122	0.0005	0.0015	0.131	9	0.0313	0.0108	0.0096	0.307
2	10	0.0120	-0.0014	0.0018	0.147	10	0.0322	0.0073	0.0108	0.335
3	10	0.0100	-0.0014	0.0020	0.196	10	0.0333	0.0122	0.0098	0.293
4	10	0.0142	-0.0039	0.0016	0.110	9	0.03	0.0049	0.0075	0.252
5	9	0.0092	0.0018	0.0020	0.221	9	0.0224	0.0120	0.0069	0.310
6	8	0.0101	0.0030	0.0073	0.722	10	0.0226	0.0339	0.0216	0.955
9	8	0.0106	-0.0020	0.0016	0.147	10	0.0283	0.0086	0.0440	0.155
12	10	0.0161	-0.0042	0.0038	0.236	10	0.0287	0.0162	0.0089	0.311
18	10	0.0183	-0.0040	0.0041	0.224	10	0.0284	0.0011	0.0055	0.195
24	10	0.0120	0.0007	0.0029	0.238	10	0.0385	-0.0175	0.0141	0.367
			Average:	0.00273	0.2297			Average:	0.01339	0.3445
			S.D.:	0.00170	0.1615			S.D.:	0.01054	0.2018
			C.V.:	62.2	70.3			C.V.:	78.7	58.6

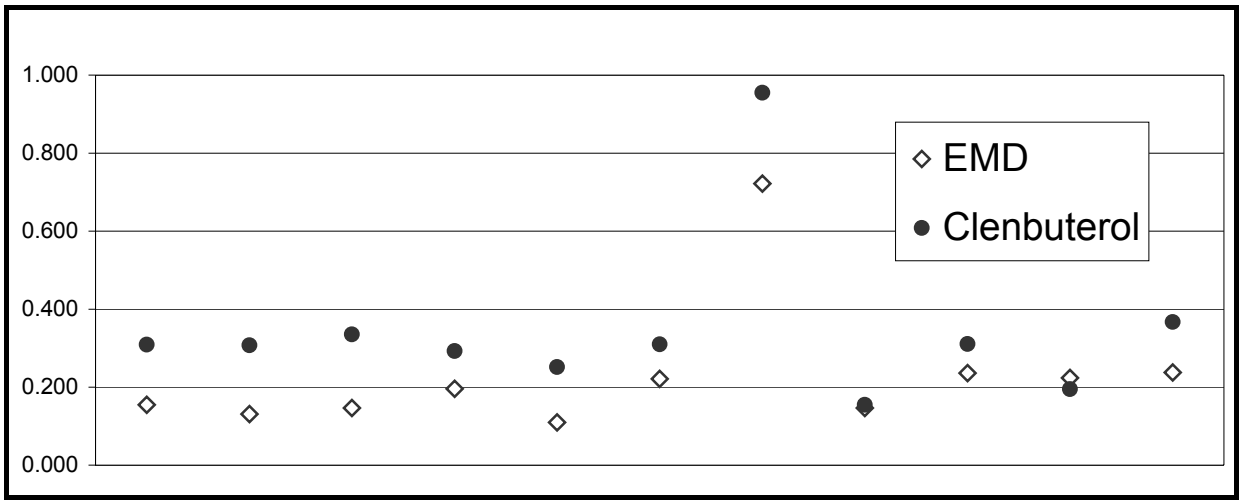

Fig 1: The standard deviations of the method for EMD and clenbuterol

Table 2: The paired t-test of the standard deviations of the method

t-Test: Paired Two Sample for Means		
	EMD	Clenbuterol
Mean	0.229727	0.344450
Variance	0.028704	0.044775
Observations	11	11
Pearson Correlation	0.940847	
Hypothesized Mean Difference	0	
Degrees of freedom (df)	10	
t Stat	-4.903991	
P(T<=t) one-tail	0.000310	
t Critical one-tail	1.812462	
P(T<=t) two-tai	0.000619	
t Critical two-tail	2.228139	

To confirm that the uncertainty of clenbuterol is **not** in principle higher the single concentrations and respective S.D.s for each temperature were calculated and these S.D.s within an analysis were averaged after removal of outliers. Table 3 shows their numerical values and the result of the t-test indicating that there are no significant differences. Table 4 shows the correctly (as per the DIN 38 402 Part 51) calculated total uncertainties u(t) treated in the same manner. The t-test confirms that the u(t) of EMD and clenbuterol are significantly different.

Table 3: Standard deviations [ng/ml] of the analysed samples and their comparison

	Clenbuterol		EMD		t-Test: Paired Two Sample for Means		
Month	n	S.D. [ng/ml]	n	S.D. [ng/ml]		*Clenbuterol*	*EMD*
1	15	0.764	15	0.410	Mean	0.388	0.301
2	14	0.144	15	0.138	Variance	0.065	0.031
3	14	0.394	15	0.575	Observations	10	10
4	15	0.332	14	0.113	Pearson Correlation	0.321	
5	14	0.317	14	0.347	Hypothesized Mean Difference	0	
6	15	0.628	14	0.426	Degrees of freedom (df)	9	
9	13	0.794	13	0.252	t Stat	1.063	
12	14	0.254	15	0.204	P(T<=t) one-tail	0.158	
18	15	0.121	15	0.046	t Critical one-tail	1.833	
24	14	0.133	13	0.498	P(T<=t) two-tail	0.316	
					t Critical two-tail	2.262	

Table 4: Total uncertainties [ng/ml] of the analysed samples and their comparison

	Clenbuterol		EMD		t-Test: Paired Two Sample for Means		
Month	n	u(t) [ng/ml]	n	u(t) [ng/ml]		*Clenbuterol*	*EMD*
1	15	0.441	15	0.167	Mean	0.460233	0.345200
2	14	0.436	15	0.194	Variance	0.079348	0.086891
3	14	0.384	15	0.264	Observations	10	10
4	15	0.335	14	0.148	Pearson Correlation	0.935893	
5	14	0.420	14	0.321	Hypothesized Mean Difference	0	
6	15	1.223	14	1.162	Degrees of freedom (df)	9	
9	14	0.215	13	0.240	t Stat	3.497538	
12	14	0.404	15	0.306	P(T<=t) one-tail	0.003375	
18	15	0.251	15	0.300	t Critical one-tail	1.833114	
24	14	0.493	13	0.351	P(T<=t) two-tail	0.006750	
					t Critical two-tail	2.262159	

Conclusion

When reference substances for calibration are very expensive or available only in small amounts calibrators are usually prepared from one solution. In such cases the uncertainty has to be extended. The data presented here shows that the normally applied multiplication with a factor 2 is sufficient to compensate for the lack of independence of the calibrators.