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Effect of Urine Matrix on the Testosterone/Epitestosterone Calibration Curve

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According to the guidelines of the International Olympic Committee testosterone administration is detected by measurement of the testosterone to epitestosterone (T/E) ratio (1). It is recommended that a calibration curve bracketing the value of the sample should be established. The epitestosterone level of the calibration should equal that of the sample. In the present study it is shown that the urine matrix influences the response factors of testosterone and epitestosterone, and it is concluded that calibrators should be prepared by adding testosterone and epitestosterone to urine instead of direct analysis of the pure compounds.

Materials and Methods

Analysis of conjugated steroids was performed as described in the literature (2, 3). In brief, conjugated steroids were extracted from 2.5 mL urine by Sep-Pak C_{18} columns, enzymatically deconjugated by ß-glucuronidase from *E. coli* (Boehringer-Mannheim), extracted into diethyl ether, and derivatized as TMS-enol-TMS ethers (75 µl MSTFA/NH_4I/DTE, 1000:2:4). 3 µl was injected with split 1:15 into the GC/MS apparatus (HP 5890 II/5970 MSD). A methylsilicone column was used (HP Ultra Performance; 12 m x 0.2 mm ID, 0.33 µm). m/z 432 was monitored corresponding to testosterone and epitestosterone, and m/z 446 for methyltestosterone, the internal standard.

Results

Table 1 shows relations between molar ratios and peak area ratios of mixtures of pure testosterone and epitestosterone analysed directly, i.e. without having been carried through the extraction procedure. For a molar ratio of one, the mean peak area ratio is also one, which means that in this case the response factors of the compounds are identical. For molar ratios of 6 and 12, however, the mean peak area ratios do not correspond to the molar ratios, but they are higher (8.6 and 20.1, respectively).

Table 1: Analysis of mixtures of testosterone and epitestosterone. Pure compounds derivatized directly

Molar ratio T/E	Area ratio T/E		
	$\bar{x}$	SEM	N
1/1	1.00	0.02	15
6/1	8.59	0.2	14
12/1	20.14	0.4	13

Epitestosterone 0.347 nmol (100 ng)
75 μl derivatizing agent
3 μl injected (split 1:15)

In order to analyse the situation in more detail, calibration curves for testosterone and epitestosterone were studied separately (Fig. 1 and 2). It is observed that the calibration curve for testosterone is non-linear, because the response (= peak area of testosterone/peak area of methyltestosterone) increases relatively more than the concentration, i.e. the response factor of testosterone increases with the concentration level. This phenomenon explains why the ratio of peak areas for testosterone to epitestosterone does not correspond to the molar ratio.

Fig. 3 shows relations between T/E calibration curves corresponding to direct analysis, T/E added to H_2O and taken through the extraction procedure, and T/E added to urine and taken through the extraction procedure. In the latter case, the enzymatic step was omitted so that the contribution from endogenous testosterone and epitestosterone was negligible. It is noticed that there is a large discrepancy between the direct curve and the curve for T/E added to urine. This means that if a direct calibration curve is used, the T/E ratio in urine samples is systematically underestimated. The degree of underestimation depends on the level, but it may easily amount to 35 % or more. The calibration curve in urine is nearly linear in opposition to the direct curve. Apparently, the urine matrix stabilizes the response factor of testosterone.

References

1. International Olympic Committee Medical Commission. Requirements for accreditation and good laboratory practices. Lausanne, 1988.

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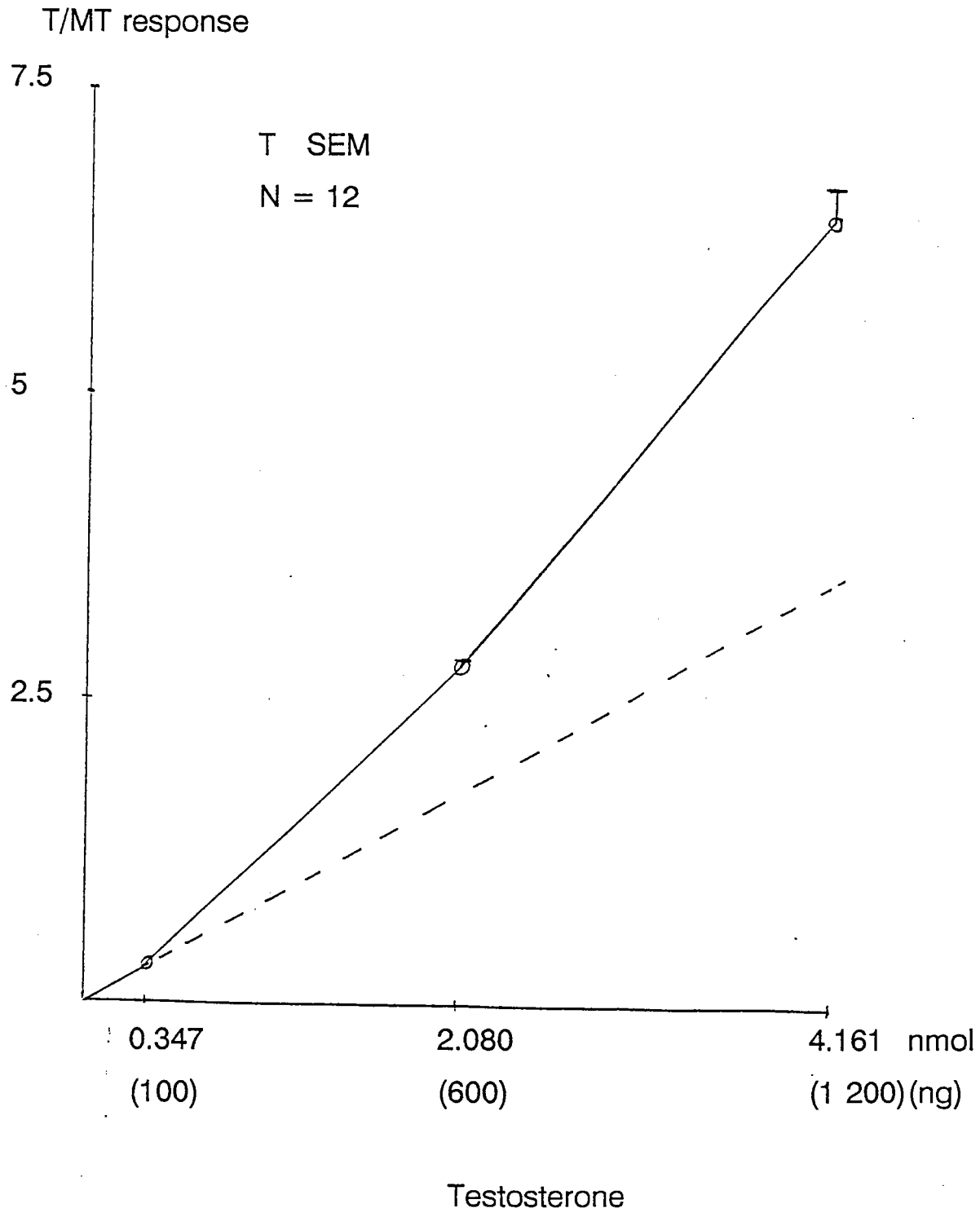

Fig. 1. Calibration curve for testosterone analyzed directly. T/MT: peak area of testosterone/peak area of methyltest.

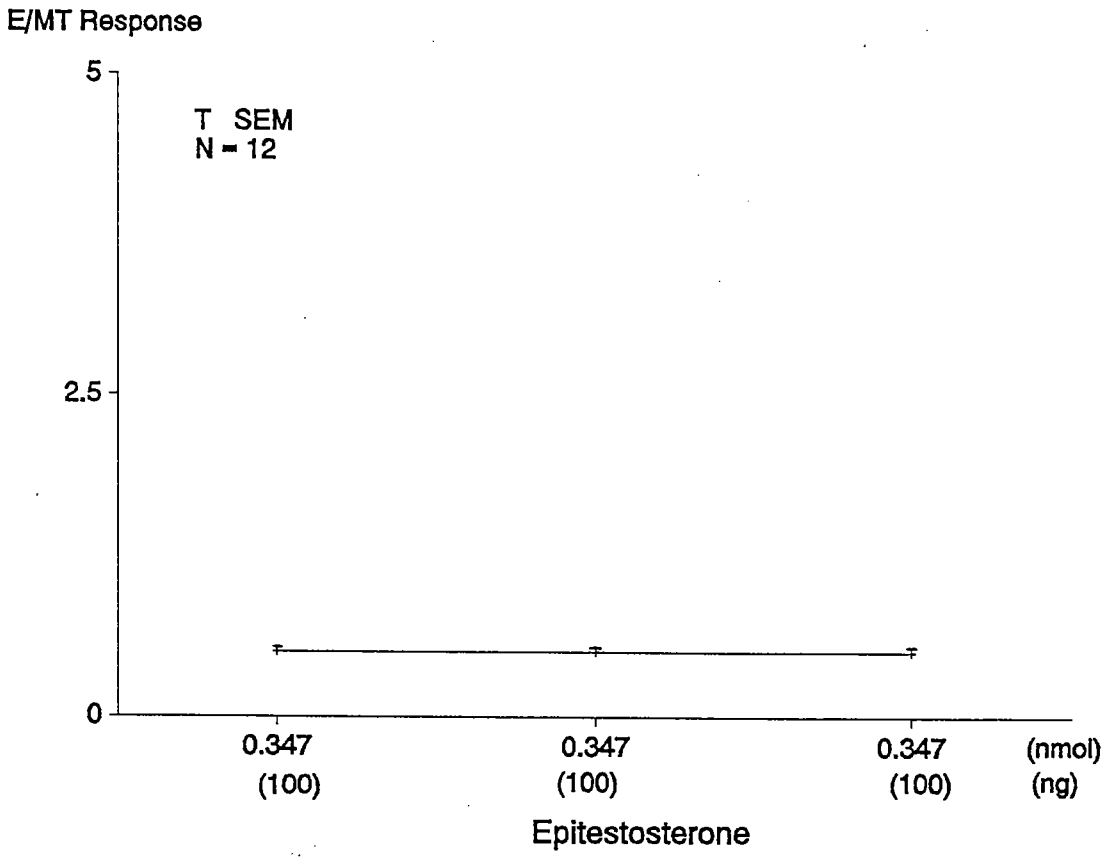

Fig. 2 Calibration curve for epitestosterone analyzed directly.

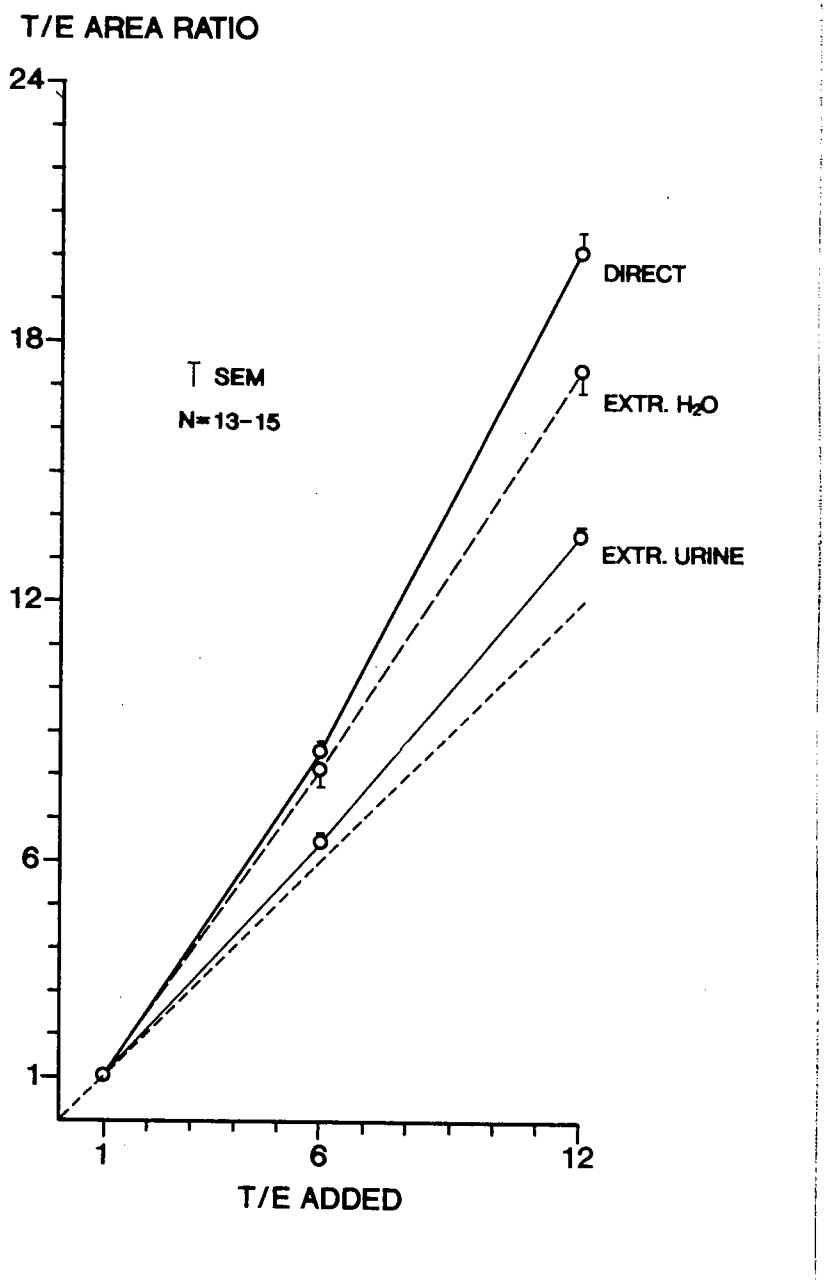

Fig. 3. T/E calibration curves corresponding to direct analysis, after addition to water and to urine.