Journal of Steroid Biochemistry, 1972, Vol. 3, pp. 325–331. Pergamon Press. Printed in Great Britain

COMPARISON AND EVALUATION OF A COMPETITIVE PROTEIN BINDING AND A GAS-CHROMATOGRAPHIC METHOD FOR THE ASSAY OF TESTOSTERONE IN PERIPHERAL HUMAN PLASMA

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SUMMARY

Plasma samples from men and women were assayed for testosterone using a gaschromatographic technique (Brownie, Van der Molen, Nishizawa and Eik-Nes, *J. clin. Endocr.* **24** (1964) 1091) and a competitive protein binding technique (essentially a modification of a method described by Mayes and Nugent, *J. clin. Endocr.* **28** (1968) 1169).

INTRODUCTION

ELECTRON capture detection of proper steroid derivatives after gaschromatography has frequently been used for the estimation of steroids isolated from human plasma[1]. The reliable estimation of small amounts of plasma steroids using g.l.c. is, however, time-consuming and occasionally of limited precision. Disadvantages of gaschromatographic techniques include the extensive prepurification of the steroid because of the non-specific detection systems. Frequently derivative formation is required before electron capture detection can be applied. Competitive protein binding analysis appeared to offer certain advantages with regard to the specificity of detection and time required for analysis[2].

We have compared the gaschromatographic technique for the estimation of testosterone in human peripheral plasma as described by Brownie *et al.*[3] with a competitive protein binding technique which is essentially a modification of the method described by Mayes and Nugent[4]. In addition to the comparison of the precision and the accuracy of the techniques, we have applied both techniques to the assay of testosterone in plasma samples of normal human females and of normal human males.

EXPERIMENTAL RESULTS AND DISCUSSION

The gaschromatographic technique using electron capture detection of testosterone chloroacetates after isolation and purification of testosterone from the plasma samples, was used exactly as described by Brownie *et al.*[3]. The internal standard used during gaschromatography was 20β-hydroxy-4-pregnen-3-one chloroacetate[5]. The accuracy and precision of this technique as used in our laboratory during the last few years does not differ from the accuracy and precision described at the time of introduction of the technique (see Ref. [3] and Tables 1 and 2). Amounts of testosterone in the order of 10 to 100 ng are estimated with a standard deviation of 10–15 per cent.

The technique using competitive protein binding analysis of testosterone isolated from plasma was a modification of the method described by Mayes and

Table 1. Accuracy and precision of testosterone estimation using g.l.c.-electron capture detection of chloroacetate[3] from October 1969 to April 1970

Testosterone added to water (ng)	*n*	Testosterone estimated (ng)	S.D. (ng)	S.D. (%)
10	18	10·1	2·5	25%
100	11	102·7	10·3	10%
Plasma pool	11	50·1	5·5	11%

Table 2. Accuracy and precision of testosterone estimation using g.l.c.-electron capture detection of chloroacetate[3] from January 1971 to August 1971

Testosterone added to water (ng)	*n*	Testosterone estimated (ng)	S.D. (ng)	S.D. (%)
10	26	7·9	1·2	13%
20	6	15·3	2·5	17%
100	23	85·0	12·5	15%

Nugent[4] (see Tables 3 and 4). Because it was not possible to achieve the low blanks which were reported in the original description of this technique, we altered the chromatographic purification steps and the elution technique. After extraction of the alkaline plasma with ether-chloroform 3:1 (v/v), the residue after evaporation of the extract was purified using thin-layer chromatography on silicagel in the solvent system chloroform-methanol 99:1 (v/v). The silicagel area on the thin-layer plate corresponding to that of authentic testosterone was eluted with ethanol according to Matthews *et al.*[16]. The residue after evaporation of the methanol was dissolved in dichloromethane and was applied to the starting line of a Whatman No. 1 paper strip. Before use the Whatman No. 1 paper strips were washed for several days in a Soxhlet apparatus with benzene-ethanol 1:1 (v/v). The chromatograms were developed in a Bush B3 system[6]. The testosterone area was eluted with methanol-dichloromethane 3:2 (v/v) according to the method described by Ganis *et al.*[17]. After the ammonium sulphate precipitation of the protein-steroid complex, the tubes were centrifuged for 10 min in the cold at 12,000 rpm (17,500 *g*).

Table 3. Competitive protein binding analysis of testosterone

1 Add to sample:
  −30000 d.p.m. [^{3}H]testosterone
  − 150 μl (4 → 100) diluted 3rd trimester pregnancy plasma
2. Incubate (2 h) at room temperature
3. Add 150 μl saturated $(NH_4)_2SO_4$ solution
4. Centrifuge (10 min) at 17,500 g in the cold
5. Take aliquot of supernatant for estimation of free ^{3}H

Table 4. Modification of a method for the estimation of testosterone in plasma using competitive protein binding analysis as described by Mayes and Nugent[4]

1. Addition of 25000 d.p.m. (0·05 ng) [^{3}H]testosterone to (1–10 ml) plasma sample
2. Ether-chloroform (3 : 1, v/v) extraction of alkaline plasma
3. Purification of testosterone by:
   a. Thin-layer chromatography, followed by
   b. Paper chromatography (Bush B3)
4. a. Sampling for counting of ^{3}H
   b. Sampling for CPB-analysis

It was possible to obtain a virtually zero blank for the analysis of water samples when known amounts of pure testosterone for the preparation of calibration curves were taken through the paper chromatographic step. This is in agreement with results discussed in a review by Nugent and Mayes[7]. The calibration curve of the per cent free testosterone vs. the nanogram amount of pure testosterone taken through the paper chromatography, as shown in Fig. 1, permits the estimation of 0·4–1·6 ng of testosterone. Analysis of water samples or preextracted plasma samples results in virtually zero blank values using this method of processed standards. The accuracy and precision of this technique for testosterone analysis, as shown in Tables 5 and 7, permits analysis of 0·5–10 ng amounts of testosterone with a coefficient of variation in the order of 10 per cent. Repeated analysis of the same plasma samples at the level of 4 and 10 ng gave a coefficient of variation of 3–5 per cent. These data are in good agreement with results for the accuracy and precision of testosterone estimation using competitive protein binding reported by other workers[4, 8–13]. Although the accuracy and precision of the gaschromatographic method may be slightly lower than that of

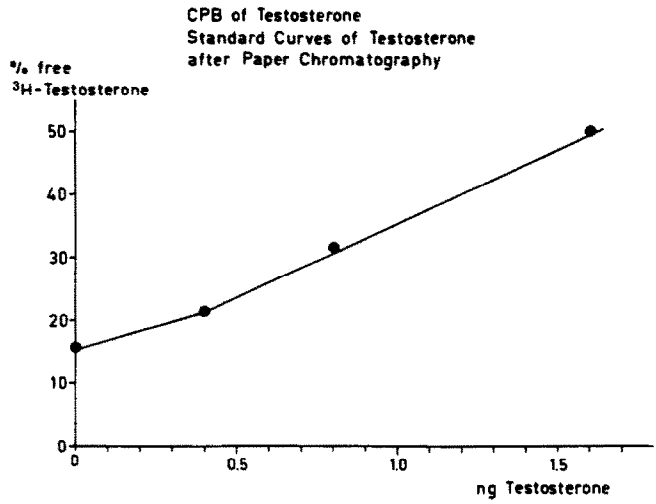

Fig. 1. Calibration curve of percent free testosterone versus ng of testosterone taken through paper chromatography. Mean values are given for repeated estimations of the protein binding of 0·4, 0·8 and 1·6 ng amounts of testosterone which were taken through the paper chromatography step (see Table 4).

Table 5. Accuracy of testosterone estimation by competitive protein binding analysis

Plasma sample no.	Testosterone estimated (ng)	Testosterone added (ng)	Testosterone estimated after addition (ng)	Recovery %
1	0·34	0·33	0·57	85·1
1	0·34	0·50	0·88	101·0
1	0·34	0·50	0·71	84·5
1	0·34	0·75	0·82	75·2
1	0·34	1·00	1·26	94·0
2	4·09	4·00	8·10	100·1
2	4·24	4·00	8·64	104·8
3	5·84	4·00	9·72	98·8
3	4·72	4·00	8·11	93·0
3	5·42	4·00	8·58	91·1
3	5·38	4·00	8·09	86·2
3	5·45	4·00	7·36	77·9
3	5·52	4·00	9·29	97·6
4	6·86	4·00	9·37	86·3
4	6·60	4·00	10·87	102·5
			mean recovery	91·9%
			S.D.	9·1%
			coefficient of variation	10·0%

Table 6. Testosterone assayed in different volumes of the same plasma samples by competitive protein binding analysis

Sample	ml plasma used	Testosterone estimated (ng/100 ml)
1	5	67
1	8	66
2	7	23
2	8	25
3	6	69
3	8	69
4	0·25	1182
4	0·50	1005
5	0·50	609
5	0·50	570
5	1·00	578
5	1·00	547
6	0·50	375
6	1·00	399
7	0·50	263
7	1·00	398

Table 7. Concentrations of testosterone in peripheral female plasma measured with a gas chromatographic technique (GLC)[3] and a competitive protein binding technique (CPB) (see Tables 3 and 4)

	Female plasma (μg/100 ml)	
	GLC	CPB
	0·013	0·023
	0·016	0·014
	0·016	0·022
	0·020	0·012
	0·029	0·018
	0·029	0·022
	0·035	0·039
	0·043	0·027
	0·093	0·038
mean	0·032	0·024
S.D.	0·025	0·010
n	9	9

correlation coefficient: 0·71
ng GLC = −11·4 + 1·84 ng CPB

Table 8. Concentrations of testosterone in peripheral male plasma measured with a gas chromatographic technique (GLC)[3] and a competitive protein binding technique (CPB) (see Tables 3 and 4)

	Male plasma (μg/100 ml)	
	GLC	CPB
	0·22	0·56
	0·30	0·35
	0·31	0·39
	0·31	0·42
	0·34	0·50
	0·37	0·45
	0·39	0·37
	0·39	0·41
	0·40	0·37
	0·58	0·37
	0·60	0·51
	0·71	0·65
	0·81	0·75
	1·01	0·88
	1·11	0·89
mean	0·524	0·525
S.D.	0·272	0·185
n	15	15

correlation coefficient: 0·869
ng GLC = −147 + 1·28 ng CPB

the competitive protein binding technique, both techniques are of low practicability. One technician can perform the analysis of approximately 12 unknown plasma samples per 5 day working week using the protein binding technique, while one technician can estimate approximately 20 plasma samples in 5 days using the gaschromatographic method.

When the same plasma samples were analysed with both the competitive protein binding technique and the gaschromatographic technique the results for analysis of female plasma samples as well as for male plasma samples (see Tables 7 and 8) show a reasonable correlation.

CONCLUSION

The gaschromatographic method as well as the protein binding method offer reliable means of estimating the small amounts of testosterone in peripheral human plasma. The precision of the protein binding method may be slightly higher, however, than the precision of the gaschromatographic method.

Although GLC and CPB methods for testosterone analysis in plasma may give comparable results, both techniques require much time and care with respect to prepurification of samples, glassware, solvents etc., and are still not very attractive for analysis of large series of samples. In this respect these results may justify the expectation that radioimmunoassay methods [14, 15] could offer a faster and simpler means of determination of testosterone isolated from biological samples.

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DISCUSSION

Munck: Why basically, is it necessary to do a preliminary chromatographic purification? Are there interfering steroids?

Van der Molen: If we want to limit the discussion to testosterone for the moment, it is necessary to separate the testosterone from compounds such as dihydrotes-

tosterone and other 17β-hydroxy androstane steroids, which will interfere with the binding of testosterone if you use the diluted pregnancy plasma as source of binding protein. It is essential when starting off with a crude extract from plasma, to use some separation technique. If one tries, as we have done, to use thin-layer chromatography throughout, impurities which are eluted from the silicagel always interfere to some extent with the protein binding system. We have only been successful with paper strips which were washed for at least 2–3 days before application. Dr. Cooke had similar experiences with a competitive protein binding assay for progesterone, namely that paper blanks, after washing of paper chromatography strips, were appreciably lower than extracts from thin-layer plates. So the necessity for chromatography *per se* is to achieve some specific isolation, and our preference for paper chromatography, at least for the last separation step, is mainly based on the lower blank values which we have obtained.

Crabbé: Dr. van der Molen, I would like to have your opinion on the reasons that, according to you, account for the rather striking slope of the regression line describing the relationship between results yielded by the two methods you've applied. One would expect a slope of one or so, but we are rather far from this kind of ratio; therefore, there appears to be a discrepancy between data going in one direction at low levels, and in the other direction at high levels. I would like to have your opinion on what, according to you, is at stake here.

Van der Molen: I have an opinion, but I do not think that it necessarily reflects the truth. Everybody working with the protein binding techniques and using diluted sera from different sources, will have to accept that the binding in these sera may differ and that at least part of the differences may reflect the lack of absolute specificity of the binding protein solution. This does not mean that I consider protein binding techniques as unspecific, but that with such small amounts in the order of 1–5 ng in the actual sample, you may lack specificity. This may also apply of course to the gaschromatographic technique.