ANALYSIS OF TESTOSTERONE AND DEHYDROEPIANDROSTERONE IN SALIVA BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT

Testosterone and 3β-hydroxyandrost-5-en-17-one (dehydroepiandrosterone) have been identified in human parotid fluid and saliva by gas chromatography-mass spectrometry/selected ion monitoring analyses of the t-butyldimethylsilyl ether and methyl oxime, t-butyldimethylsilyl ether derivatives. High specificity of analysis has been achieved by the use of high mass spectrometric resolution or by the monitoring of metastable peaks. Quantitative analyses indicate concentrations of both unconjugated testosterone and unconjugated dehydroepiandrosterone in the range 200-800 pmol/l in the saliva and parotid fluid of the normal males examined. These represent 1.5-7.5% of the concentrationsof the steroids in blood plasma taken from the same subjects.

INTRODUCTION

The current interest in the assay of salivary steroids is attributable in part to the advantages of a non-invasive, and hence stress-free, sampling technique (1,2). Furthermore, the available data suggest that the well-substantiated correlation of salivary concentrations of many drugs with the concentrations of the non-protein bound forms in blood plasma (3,4) may also be applicable to steroids (5). Thus, salivary steroid assays may provide information of greater diagnostic value than analyses of plasma, where significant changes in the concentrations of a physiologically active steroid may be obscured by relatively high concentrations of its protein-bound form.

The low concentrations of steroids in mixed and parotid saliva severely restrict the number of applicable analytical methods. Immunoassay techniques have been applied (1,2,5,6,7) and will doubtless continue to be the methods of choice for routine use. The unequivocal identification of steroids in saliva, however, requires the application of alternative highly selective techniques which may also be used to validate quantitative data obtained by the routine methods. Analytical procedures incorporating gas chromatography-mass spectrometry (GC-MS) with selected ion monitoring (SIM) are now widely accepted as reference methods for the determination of steroids in blood (8,9,10). The refinements of high mass spectrometric resolution (11, 12) or of monitoring metastable peaks (13) further enhance analytical specificity whilst retaining high sensitivity. This paper reports the application of these techniques to the analysis of unconjugated testosterone and 3β-hydroxy-androst-5-en-17-one (dehydroepiandrosterone; DHA) in saliva. Preliminary aspects of the work have been reported elsewhere (12).

METHODS

Collection of samples

Blood samples were collected by venepuncture. Parotid fluid was obtained by fitting a Curby cup over the duct of the parotid gland; secretion was stimulated by administration of citric acid on the tongue, as described elsewhere (1). Saliva was obtained after subjects had rinsed the mouth with water and a minimum of 15 min had elapsed. All samples were collected in glass tubes and stored at -20°C until use. Saliva samples were centrifuged prior to extraction; solid material was discarded.

Extraction and fractionation of extracts

Saliva or parotid fluid (5-7.5 ml) and plasma (1-2 ml) samples were twice extracted with diethyl ether (15 ml). Where applicable, total extracts of aliquots of a single fluid sample were combined. Solvent was removed under a stream of nitrogen and the residue dissolved in 200 µl of methanol:water:chloroform (9:1:2, by vol.; solvent A) and applied to a column (4 cm x 0.5 cm) of Lipidex 5000 (Packard Instruments, Downers Grove, IL 60515, U.S.A.)

swollen in solvent A (14). A 0-2 ml eluate fraction was collected. Solvent was removed under nitrogen and the residue dissolved in hexane:ethanol (4:1, by vol.; solvent B). The solution was applied to a column (4 cm x 0.5 cm) of Sephadex LH-20 (Pharmacia, Uppsala, Sweden), swollen in solvent B; a 0-4 ml fraction was collected and solvent again removed under nitrogen. Recoveries from each gel chromatographic separation exceeded 92%, as judged by model experiments with radiolabelled steroids. For quantitative analyses, samples of blood plasma (1-2 ml) or saliva (10-20 ml) were supplemented with a solution of 17α-hydroxy-androst-4-en-3-one (17-epitestosterone; 5 ng) in 10 µl ethanol. After mixing and equilibration overnight, extraction and extract purification were performed as described above.

Derivatisation

t-Butyldimethylsilyl (TBDMS) ethers were prepared by dissolving the steroid (or fraction of an extract of a biological sample) in t-butyldimethylchlorosilane : imidazole : dimethylformamide (1:1:6, by vol.; Applied Science Laboratories, State College, PA 16801, U.S.A.) and allowing to stand overnight at room temperature. Excess reagent was removed by passage through a column (2 cm x 0.5 cm) of Sephadex LH-20, swollen in hexane : chloroform : methanol (10:10:1, by vol.) (15). Methyl oximes were prepared, before or after TBDMS ether formation, by dissolving the steroid in 20 µl of a solution of methoxyamine hydrochloride (Eastman Kodak, Rochester, NY 14650, U.S.A.) in pyridine (15 mg ml^{-1}) and heating at 60° for 1 h. Pyridine was removed under a stream of nitrogen. All samples were dissolved in ethyl acetate for GC-MS analysis.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyses were performed using a Varian 2700 gas chromatograph coupled to a Varian MAT 731 double-focusing mass spectrometer via a two-stage Watson-Biemann separator. Separations were achieved on glass columns (2 m or 3 m x 3.5 mm, i.d.) of 1% OV-1, 1% OV-17, or 3% Poly-S 179 on Gas Chrom Q (100 - 120 mesh). The electron energy was 70 eV and the ion source temperature was 200°. For selected ion monitoring at high mass spectrometric resolution ($m/\Delta m$ 8500-12000, 10% valley definition), ions of chosen exact mass were focused, using the peak matching unit, by reference to ions derived from perfluorokerosene (Pierce Chemical Co., Rockford, IL 61105, U.S.A.), which was independently introduced into the ion source. For metastable peak monitoring analyses of methyl oxime t-butyldimethylsilyl ether derivatives (16), daughter ions of m/z 374 were focused at an accelerating voltage of 8 KV. The accelerating voltage was subsequently increased, with constant electric sector voltage, to locate the metastable peak corresponding to the transition, m/z 431 → m/z 374, occurring in the first field-free region. The

mass spectrometric resolution was 1000.

RESULTS AND DISCUSSION

Table 1 records salient features of the mass spectra (electron impact; 70 eV) of the t-butyldimethylsilyl (TBDMS) ethers and methyl oxime (MO) TBDMS ethers of testosterone, DHA and isomeric steroids. In all cases, $|M-C_4H_9|^+$ ions constitute the base peaks and comprise a high proportion of the total ion current. Selected ion monitoring (SIM) of these ions therefore affords analyses of high sensitivity; detection limits of 20-50 pg are obtained during GC-high resolution MS/SIM of standard compounds. Molecular ions of TBDMS derivatives of steroids are not generally observed at significant intensities (15, 17) but the MO, TBDMS derivatives of testosterone and 17-epitestosterone are exceptions (Table 1). In these instances, molecular ions are presumably stabilised by the 4-ene, 3-methyl oxime system. Intense metastable peaks are observed corresponding to the fragmentation, $M^{+\cdot} \rightarrow |M-C_4H_9|^+$

Table 1. Mass spectrometric data for derivatives of testosterone, dehydroepiandrosterone (DHA) and isomeric steroids.

Steroid	Mass Spectrum (relative intensities)		
	$M^{+\cdot}$	$\|M-C_4H_9\|^+$	$\|M-C_4H_9-H(CH_3)_2SiOH\|^+$
TBDMS ethers	m/z 402	m/z 345	m/z 269
testosterone	<1	100	6
17-epitestosterone	<1	100	27
DHA	<1	100	4
3α-hydroxyandrost-5-en-17-one	<1	100	21
MO, TBDMS ethers	m/z 431	m/z 374	m/z 298
testosterone	52	100	31
17-epitestosterone	55	100	69
DHA	<1	100	5
3α-hydroxyandrost-5-en-17-one	<1	100	10

occurring in the first field-free region of the double-focusing mass spectrometer (16). GC-MS with metastable peak monitoring of MO, TBDMS derivatives of testosterone and 17-epitestosterone yields detection limits of ca. 30 pg. In this mode of analysis, detection of DHA and 3α-hydroxy-androst-5-en-17-one is, as expected (Table 1), less sensitive by at least a factor of 10. Loss of the elements of dimethylsilanol from $|M-C_4H_9|^+$ ions represents the only other significant fragmentation pathway observed for these derivatives (Table 1).

An extract of pooled mixed saliva from a normal male was purified by gel chromatography and converted to the TBDMS derivative. Fig. 1A shows the trace obtained by GC-high resolution MS with SIM of m/z 345.2250 ($|M-C_4H_9|^+$ for testosterone TBDMS and isomeric steroids). Intense peaks were observed at retention times characteristic of testosterone and DHA derivatives. The remaining sample was converted to the MO, TBDMS ether and analysed by GC-high resolution MS with SIM of m/z 374.2515 ($|M-C_4H_9|^+$ for testosterone MO, TBDMS and isomeric steroids). Two peaks were again observed at retention times corresponding to testosterone and DHA (Fig. 1B). Analyses of the same samples using GC columns of differing selectivity (OV-1, OV-17 and Poly S-179) in each case afforded peaks at the appropriate retention times.

An extract of saliva taken from the same subject on a different occasion was analysed as the MO, TBDMS derivative with SIM at high mass spectrometric resolution (Fig. 2A) and by metastable peak monitoring (Fig. 2B). In the latter analysis, a single peak was observed at a retention time corresponding to testosterone MO, TBDMS; as expected, DHA MO, TBDMS was not detected during metastable peak monitoring.

Further evidence for the identification of testosterone and DHA in saliva was obtained following addition of 17-epitestosterone as internal standard. Purified extracts

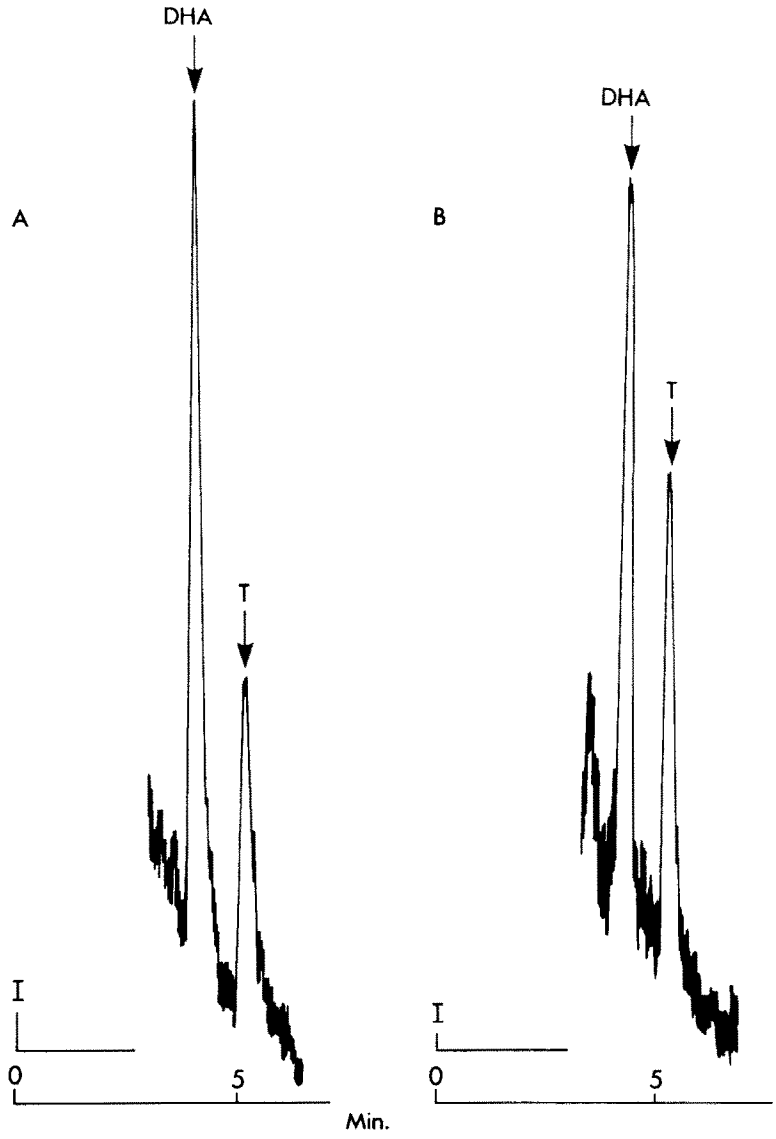

Fig. 1: GC-high resolution MS/SIM analyses of aliquots of a purified extract of mixed saliva (40 ml). A. TBDMS ether; m/z 345.2250. GC conditions : 1% OV-1, 245°. Analysis of 10% aliquot. B. MO, TBDMS ether; m/z 374.2515. GC conditions : 1% OV-1, 255°. Analysis of 15% aliquot.

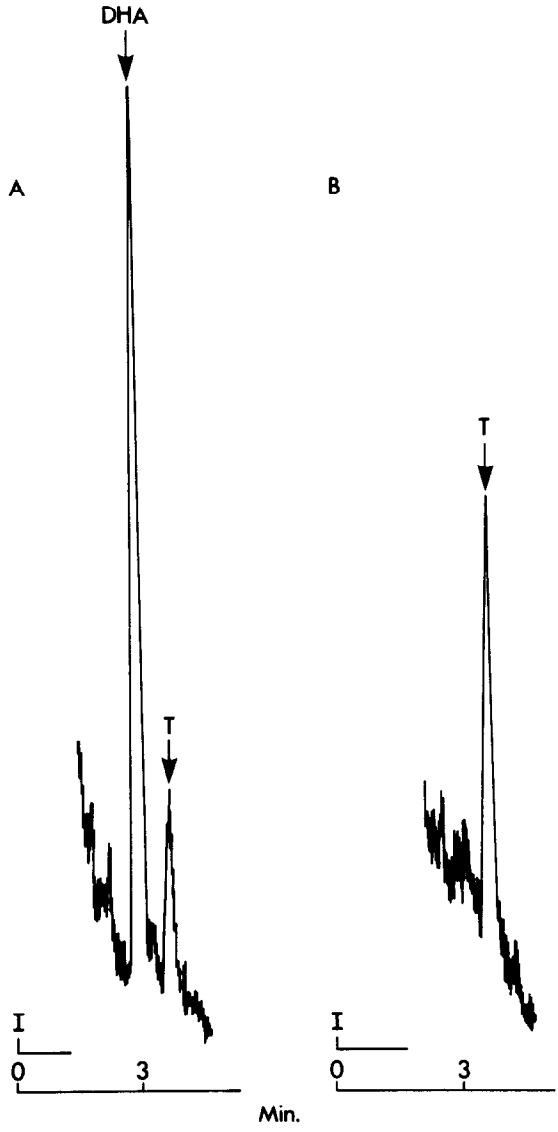

Fig. 2: GC-MS analyses of aliquots of the MO, TBDMS derivative of a purified extract of mixed saliva (40 ml). A. SIM of m/z 374.2515. B. metastable peak monitoring of m/z 431 → m/z 374. GC conditions : 1% OV-1, 255°.

were analysed as TBDMS ethers with SIM at mass spectrometric resolutions of 8,500 and 12,000. The ratios of the intensities of the peaks attributable to testosterone and DHA to the intensity of the 17-epitestosterone peak were unchanged at the higher resolution, confirming that the ions detected were indeed of the exact mass monitored (rather than of a closely similar, and incompletely separated, mass).

The addition of 17-epitestosterone internal standard also enabled quantitative estimation of testosterone and DHA by GC-MS/SIM of TBDMS derivatives. Table 2 records concentrations of the two steroids in matched samples of blood plasma and mixed or parotid saliva from male subjects (25-30 years). Salivary concentrations of testosterone are in accordance with data obtained by enzyme- and radioimmunoassays (5,6,7), substantiating the specificity of the routine immunoassay procedures. Walker et al., for example, reported radioimmunoassay data which indicated morning concentrations of testosterone of 0.37 $\pm$ 0.17 nmol/l and evening concentrations of 0.21 $\pm$ 0.13 nmol/l in the saliva of normal males (7). The analysis of DHA in saliva has not been reported previously. For both steroids, the salivary concentrations represent 1.5-7.5% of the concentration in blood plasma. This is consistent with the hypothesis that salivary steroid concentrations correspond to the concentrations of the non-protein bound form in blood plasma (5). The present limited data, however, do not permit an assessment of the correlation of plasma and saliva concentrations.

CONCLUSION

Highly specific techniques of GC-MS have been applied to the identification of testosterone and dehydroepiandrosterone in mixed and parotid saliva. Quantitative determinations by GC-MS are in accordance with recently reported immunoassay data. The application of GC-high resolution MS/SIM to the rigorous validation of routine immunoassay procedures is in progress and will be reported elsewhere.

Table 2. Concentrations of testosterone and dehydroepiandrosterone in blood plasma, mixed saliva and parotid saliva of male subjects.

Subject	Testosterone (nmol/l)*			DHA (nmol/l)*		
	Plasma	Mixed Saliva	Parotid Saliva	Plasma	Mixed Saliva	Parotid Saliva
A1**	20.7(19.3)	0.364	-	7.12(6.77)	0.528	-
A2**	21.8	-	0.427	18.6	-	0.465
B	13.2(11.5)	0.243	-	6.88(6.94)	0.208	-
C	9.20	0.271	-	14.9	0.333	-
D	34.2	-	0.486	15.4	-	0.809

* Values in parentheses are from duplicate determinations.

** Samples from the same subject taken on different occasions.

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