Journal of Chromatography, 89 (1974) 215–224

CHROM. 7108

DETERMINATION OF PLASMA TESTOSTERONE BY COMBINED GAS CHROMATOGRAPHY–MASS SPECTROMETRY

J. R. CHAPMAN
A.E.I. Scientific Apparatus Limited, Manchester (Great Britain)
and
E. BAILEY
Medical Research Council Unit for Metabolic Studies in Psychiatry, Middlewood Hospital, Sheffield (Great Britain)
(Received September 6th, 1973)

SUMMARY

A rapid quantitative method has been developed for the estimation of testosterone in human male peripheral plasma using the technique of combined gas chromatography–mass spectrometry. Plasma samples are submitted to a simple extraction procedure and after forming the bromomethyldimethylsilyl ether derivative are analysed without further purification on a 50 cm 2.0% OV-1 column. Deuterium-labelled testosterone is added to the plasma as an internal standard and quantitation effected by multiple peak monitoring at low resolving power. The standard deviation of the method is 7.2% of the mean value for a determination at the level of 0.81 μg/100 ml plasma. In addition, it is shown that the method has sufficient inherent sensitivity to be applicable to the estimation of testosterone in female plasma. In an alternative technique, after a preliminary thin-layer separation, the same samples are introduced directly into the mass spectrometer source and analysed at a higher resolving power. An analysis of the limitations imposed by the purity of the stable isotope-labelled compound used as internal standard in multiple peak monitoring methods is also presented.

INTRODUCTION

The estimation of testosterone in human peripheral plasma using the technique of double isotope dilution[1–3] or gas–liquid chromatography (GLC) with electron capture detection[4–6] is both time-consuming and tedious. Competitive protein binding methods[7–9] and the recently developed radioimmunoassay methods[10,11] offer a more practical and reliable means of estimating this plasma steroid but these still involve one or more chromatographic purification steps and difficulties are often encountered with high non-specific blank values.

The object of this investigation was to develop a more rapid assay procedure

for male plasma testosterone using combined gas chromatography–mass spectrometry (GC–MS). This technique with multiple peak monitoring (mass fragmentography) is capable of estimating compounds with a high degree of specificity and sensitivity[12,13] and is therefore ideally suited to the estimation of nanogram quantities of steroids isolated from biological fluids[14,15]. In the method to be described deuterium-labelled testosterone is added initially to the plasma as an internal standard. This approach of employing an isotopically labelled compound for this purpose has recently been effectively used to measure aldosterone in plasma[16], and a number of other compounds isolated from biological fluids in low concentrations by multiple ion monitoring[17–20].

In a previous paper[21] we reported on the suitability of the chloromethyldimethylsilyl ethers as derivatives for the estimation of hydroxylated steroids by single or multiple peak monitoring techniques. Subsequently we have found that the bromomethyldimethylsilyl (BDMS) ether of testosterone has additional advantages as a derivative for the quantitative estimation of this particular steroid.

MATERIALS AND METHODS

Steroids

Testosterone was obtained from Steraloids (Croydon, Great Britain), and its purity confirmed by GLC and GC–MS. Crystalline deuterium-labelled testosterone was prepared as follows: 100 mg testosterone was dissolved in 1 ml tetrahydrofuran in a screw-capped tube with a PTFE-lined cap. 1 ml deuterium oxide (Isotopic Purity 99.8% Atom % D from Ryvan, Southampton, Great Britain) and several milligrams of anhydrous potassium carbonate were added and the mixture allowed to react at 100° for 48 h. The solution was then taken to dryness on a rotary evaporator and redissolved in ether. The ether extract was washed with distilled water, dried over anhydrous sodium sulphate and then taken to dryness. The final product was recrystallized twice from methanol and its purity confirmed by thin-layer chromatography (TLC) and by GLC and GC–MS, both as the free steroid and its BDMS ether derivative.

Chemicals

All solvents were obtained from British Drug Houses (Poole, Great Britain) and were of reagent grade unless otherwise stated. Hexane was washed with conc. H_2SO_4 and water, dried over anhydrous sodium sulphate, and redistilled. Diethylamine was redistilled and stored over KOH pellets. Diethyl ether (AnalaR grade) was redistilled before use. Bromomethyldimethylchlorosilane (Pierce, Rockford, Ill., U.S.A.) was redistilled and stored in sealed ampoules at 0°.

Procedure

A 4-ml volume of heparinized male plasma was diluted to 10 ml with distilled water and 0.5 ml 2 *N* NaOH was added. From a standard solution containing 1 ng/μl deuterium-labelled testosterone in ethanol, 100 μl was added to the plasma sample. The diluted plasma was extracted with 30 ml of diethyl ether three times and the combined extracts washed twice with 5 ml distilled water, dried over anhydrous sodium sulphate, and then evaporated to dryness on a rotary evaporator. Standards

and extracts were reacted to form the BDMS ethers using the procedure described previously[21]. The derivatized extracts were finally taken up into 0.2 ml hexane for analysis by multiple peak monitoring.

Gas chromatography–mass spectrometry

The instrument used was an MS-30 double-beam, double-focussing, mass spectrometer (AEI, Manchester, Great Britain) equipped with a multiple peak monitoring system capable of operating at low and high resolving powers. With this system up to six separate masses can be monitored by switching the mass spectrometer accelerating voltage at a speed of 0.25 sec per channel. The signal detected at each mass is integrated over the dwell time and the integrated values held during the rest of the switching cycle. The resulting stepped signals are then smoothed to provide continuous traces for each channel. Each channel has an independent output and its own gain and backing off controls. The output can be displayed either on a UV galvanometer recorder using a low paper speed or on a multiple pen recorder.

The gas chromatograph (Pye Series 104; Pye Unicam, Cambridge, Great Britain) was interfaced to the mass spectrometer using a single-stage membrane separator[22]. The quantitative analyses of male plasma extracts were carried out on a 50 cm × 4 mm I.D. glass column packed with 2% OV-1 coated on 100–120 mesh Gas-Chrom Q. The column was operated at 240° with a helium flow-rate of 45 ml/min. The analysis of the female plasma extract and the comparative analyses of the male plasma extract at high and low resolving power were carried out on a 50 cm × 4 mm I.D. glass column packed with 2% OV-225 coated on 100–120 mesh Gas-Chrom Q. This column was programmed from 240–270° at 4°/min with a helium flow-rate of 40 ml/min. The mass spectrometer was operated under the following conditions: full accelerating voltage, 4 kV; nominal electron energy, 70 eV; ion source temperature, 200°; and molecular separator temperature, 230°. The instrument was adjusted to monitor masses 438, 440 and 442 at a resolving power of 1000. Initial setting up at these nominal masses was accomplished by using the second beam containing perfluorokerosene reference compound. Final setting up at the correct fractional masses to monitor the testosterone derivative was accomplished by introducing the standard deuterated testosterone BDMS ether which also contains a significant amount of the non-deuterated material into the mass spectrometer source. Monitoring of a selected reference mass in the second beam during analyses can be used to indicate and allow correction for any drift in magnet current. Adjustment of the potentiometers which determine the separate masses monitored by voltage switching was not found to be necessary during the analyses.

The standard curves used for the estimation of testosterone in male plasma were prepared using the deuterium-labelled testosterone as the internal standard. Increasing amounts of testosterone ranging from 10 to 80 ng were added to 4-ml aliquots of distilled water, and these were then taken through the assay procedure described for plasma samples. The peak height ratio between *m/e* 438 and *m/e* 442 was plotted against amount of testosterone (in ng) added and a straight line, from which the concentration of testosterone in the plasma sample could be measured, fitted by the usual least squares procedure.

RESULTS AND DISCUSSION

The BDMS ether derivative of testosterone is readily formed in quantitative yield and has good GLC properties. It is stable in hexane and extracts containing testosterone BDMS ether can be stored at 0° for several months without loss. The mass spectrum of testosterone BDMS ether is illustrated in Fig. 1a. The high intensity of the molecular ion at *m/e* 438 meets one of the most important criteria in choosing

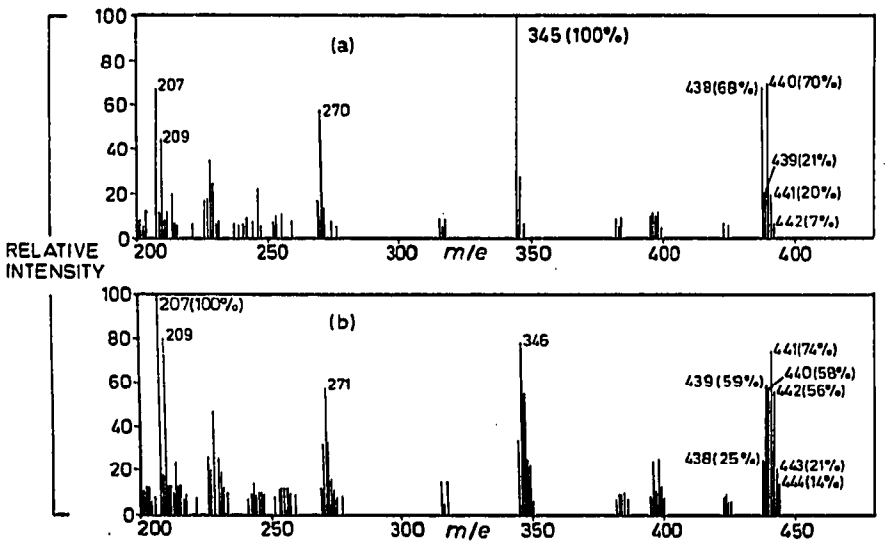

Fig. 1. Mass spectra of the BDMS ethers of (a) reference testosterone and (b) deuterated testosterone used as internal standard.

derivatives for multiple peak monitoring work, *i.e.* the presence of intense ions at high mass where interference from instrumental background, column bleed or other components in the sample being analysed is at a minimum. The presence of bromine in the molecule provides a characteristic isotopic pattern; thus, monitoring both molecular ions at *m/e* 438 (^{79}Br) and *m/e* 440 (^{81}Br) and determining the ratio of their intensities provides a check on the absence of contributions from other materials at these masses. The base peak at *m/e* 345 in the spectrum is due to the elimination of CH_2Br from the molecular ion and the fragment ion at *m/e* 270 results from the elimination of the alcohol moiety.

The mass spectrum of the deuterated testosterone BDMS ether (Fig. 1b) shows the major components of the standard are d_0-, d_1- and d_2-testosterone, in the approximate ratio 1:2:1, together with smaller amounts of d_3- and d_4-testosterone. Measurement of the internal standard at *m/e* 442 and the testosterone at *m/e* 438 enabled a linear calibration plot (Fig. 2) to be drawn over the concentration range required for determinations in male plasma (see Appendix) as well as providing peaks of adequate intensity for quantitative determination.

Typical multiple peak monitoring traces from (a) standard testosterone BDMS ether, (b) a male plasma extract, and (c) a male plasma extract containing the

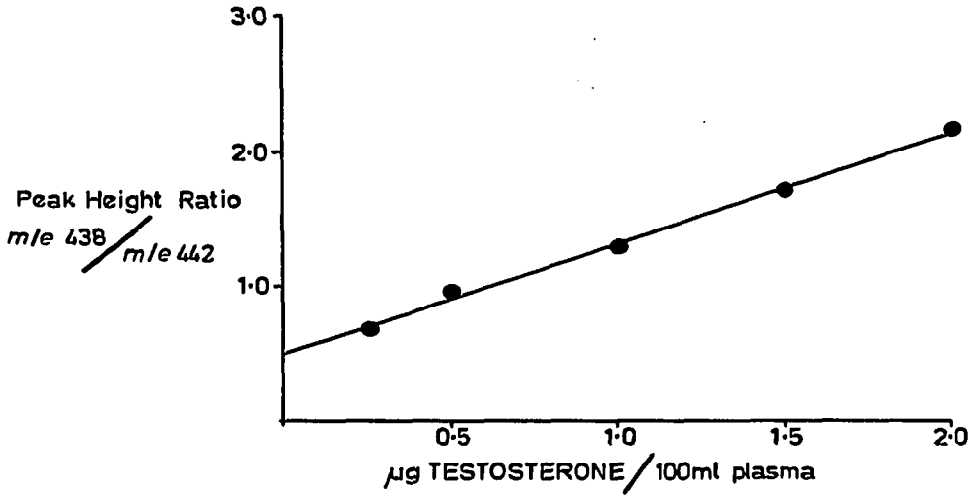

Fig. 2. Standard curve used for the quantitative determination of testosterone in male plasma.

deuterated internal standard are shown in Fig. 3. A comparison of the ratio of the peak heights at *m/e* 438 and *m/e* 440 in the plasma extract (Fig. 3b) with the same ratio in the standard (Fig. 3a) confirms the absence of contributions from other materials at these masses in the plasma extract. A similar comparison of the ratio of the peak heights at *m/e* 438 and *m/e* 442 again in the plasma extract with the same ratio in the standard shows that the extract does not have a measurable additional contribution at *m/e* 442 which would interfere with the measurement of the deuterated standard at this mass. Thus, by initially monitoring the plasma extract without internal standard, it is possible to confirm that other materials in the extract will not interfere with the accurate determination of testosterone levels. Extracts which do not meet these criteria are not suitable for further analysis.

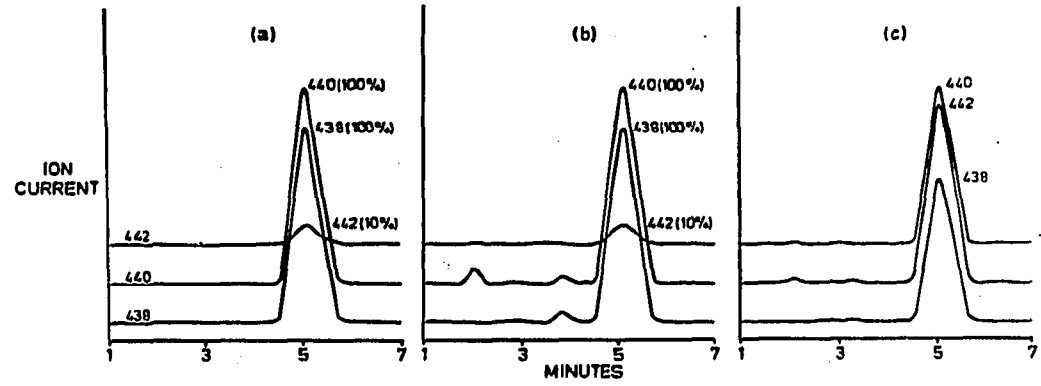

Fig. 3. Multiple peak monitor traces from (a) standard testosterone BDMS ether (20 ng injected), (b) male plasma extract without internal standard (equivalent of 0.2 ml plasma injected), and (c) male plasma extract containing deuterated internal standard (equivalent of 0.2 ml plasma injected). The retention time of testosterone BDMS ether is 5.1 min. The small peaks in traces (b) and (c) with a retention time of 3.8 min correspond to dehydroepiandrosterone BDMS ether.

The suitability of the GC–MS system for the analysis of such steroid derivatives at low levels (analysis of a male plasma in our system typically involves the injection of a total of 5–8 ng of testosterone BDMS ether in the labelled and unlabelled forms on the GLC column) is illustrated in Fig. 4. This figure shows the response recorded at *m/e* 438 from decreasing quantities of standard testosterone BDMS ether injected into the GC–MS system. The linear variation of this response with sample quantity demonstrates that testosterone BDMS ether can in fact be analysed at the sub-nanogram level without measurable losses due to adsorption in either the GLC column or the GC–MS interface.

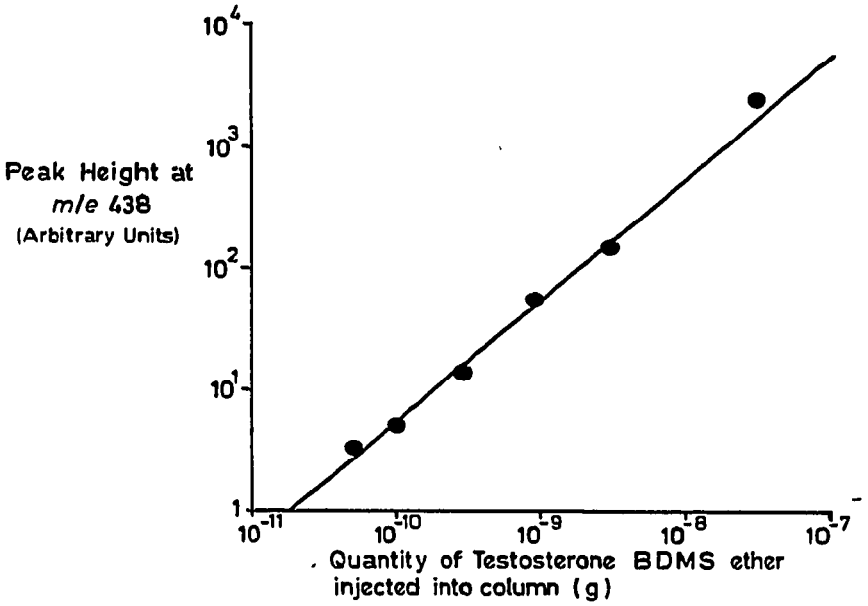

Fig. 4. Single peak monitor response (measured as peak height at *m/e* 438) obtained from decreasing quantities of standard testosterone BDMS ether injected.

The precision of the method was determined by performing six replicate analyses on a pool of male plasma. The mean of the six determinations was 0.81 μg testosterone/100 ml plasma and the standard deviation was 7.2% of this mean value. The plasma testosterone level was also measured in six single plasmas taken from normal males aged 20–40 years. The values obtained, which ranged from 0.42 to 1.37 μg/100 ml plasma, are similar to those reported in the literature using alternative techniques.

The single ion monitor trace at *m/e* 438 from a female plasma extract (Fig. 5a) not containing internal standard illustrates the inherent sensitivity of the method. Female plasma testosterone levels are, on average, an order of magnitude lower than those in male plasma so that less internal standard should be added to female plasma as compared with male plasma (see Appendix). However, to maintain the same order of accuracy as obtained in quantitative determinations on the male plasma, a corre-

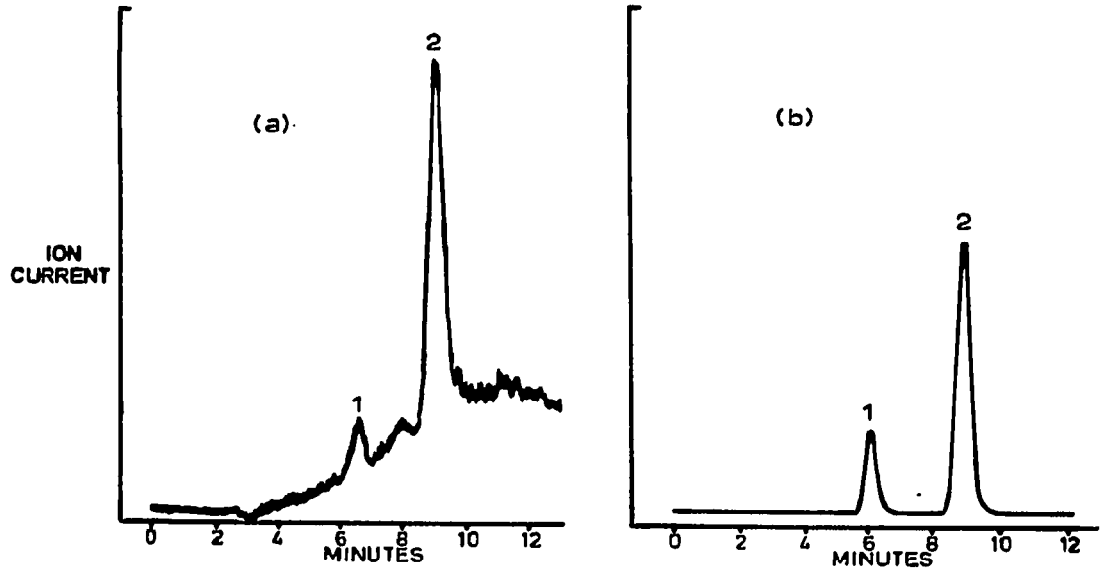

Fig. 5. Single peak monitor traces recorded at *m/e* 438 from (a) female plasma extract without internal standard (equivalent of 0.3 ml plasma injected), and (b) mixture of standard dehydroepiandrosterone BDMS ether (2 μg injected) and testosterone BDMS ether (1 μg injected). The retention time of dehydroepiandrosterone BDMS ether is 6.7 min and that of testosterone BDMS ether is 9.2 min. 1 = Dehydroepiandrosterone; 2 = testosterone.

spondingly larger fraction of the extract would then have to be loaded on to the GLC column, preferably by a solid-loading technique.

In order to extend the method so that one standard procedure using a labelled internal standard would cover testosterone determination in both male and female plasma, use of the correction techniques described in the Appendix with perhaps some improvement in the purity of the internal standard would be necessary. Extension of the method to testosterone determination in female plasma by means of the improvements outlined above is now under investigation.

The analysis shown in Fig. 5a was carried out on a 2% OV-225 column and the rise in recorder base line during this run is caused by an increase in bleed from this stationary phase. Interference from column bleed or from other components in the sample limits the available sensitivity of the method so that although the OV-225 column provides a better separation of dehydroepiandrosterone and testosterone BDMS ethers (Fig. 5b) it was replaced by the more thermally stable non-polar OV-1 column for use in quantitative analyses. The effects of column bleed can also be eliminated by directly introducing the sample into the ion source on a solid probe. Use of a direct introduction technique could also confer the advantages of much greater speed of operation compared with GC–MS for routine samples, perhaps by a factor of ten for the testosterone assay described in this paper. However, the use of the mass spectrometer at a higher resolving power would be necessary because of the loss of the GLC separation of interfering sample components. A resolving power of 3000 was found to be sufficient to separate the mass deficient testosterone BDMS ether (molecular weight 438.1590) from other impurities. For example, the fractional mass

of a saturated hydrocarbon fragment at this mass is approximately 438.4. Thus, the small loss of sensitivity caused by the increase in resolving power can be more or less completely offset by the efficient means of sample introduction. The application of this high resolution technique to plasma testosterone determinations would also necessitate a preliminary TLC separation, after addition of the internal standard, to remove components such as dehydroepiandrosterone which could otherwise not be distinguished from testosterone. However, this TLC separation could easily be carried out routinely on a batch basis. Experiments carried out so far have demonstrated the efficient separation of impurity peaks at a resolving power of 3000. Fig. 6a shows a single peak monitoring trace obtained from a badly contaminated plasma extract run at a resolving power of 1000. For this trace and that shown in Fig. 6b the extracts

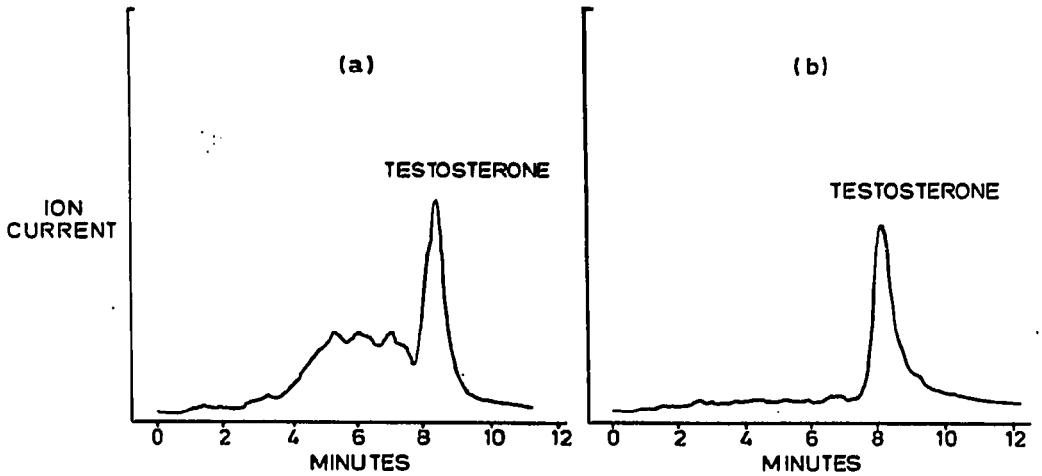

Fig. 6. Single peak monitor traces recorded at *m/e* 438 from a male plasma extract using (a) a resolving power of 1000, and (b) a resolving power of 3000 (multiplier gain increased to maintain height of testosterone peak).

were in fact run on the GC–MS to demonstrate the presence of interfering materials that co-distil with testosterone BDMS ether using the solid probe. At 1000 resolving power these materials would then be indistinguishable from testosterone BDMS ether but, as can be seen in Fig. 6b, at a resolving power of 3000 these materials no longer interfere.

APPENDIX

Whilst stable isotope-labelled compounds provide ideal internal standards for quantitative determinations using multiple peak monitoring methods, it is often impractical to prepare these in other than moderate isotopic purity. It is therefore important to define the limitations imposed by the use of simply prepared but less pure labelled standards such as that used in this work. Let the mass at which the sample is measured be M_1 and that at which the standard is measured be M_2. Let the intensities of the contributions at M_1 and M_2 from the pure sample be a_1 and a_2, respectively,

and those from an equal quantity of the standard be b_1 and b_2, respectively. If, in any run, the respective amounts of sample and standard are A and B, the measured ratio, R, is then given by the expression:

$$R = \frac{a_1A + b_1B}{a_2A + b_2B} \quad (1)$$

The limitations imposed by the use of impure standards arise from two sources:

(i) interference from the sample at mass M_2. Ideally this interference is negligible, *i.e.* $a_2A \ll b_2B$ in eqn. 1, and a linear calibration plot ensues, *i.e.*

$$R = \left[\frac{a_1}{b_2B}\right]A + \frac{b_1}{b_2}$$

However, if the standard is less pure, b_2 is smaller so that the product a_2A cannot now be neglected. R is no longer a linear function of A and the use of a straight-line calibration plot under these circumstances introduces an error into concentrations determined from the plot. The practical effect of this interference can be illustrated by reference to the standard and calibration plot (Fig. 2) used in the present work. For the determination of testosterone over a concentration range of 0.375–2.000 μg/100 ml plasma, a maximum error, due to non-linearity, of 2.7% is introduced. If the same calibration is extended to include higher concentrations giving an effective range of 0.375–5.500 μg/100 ml, then the maximum error increases to 48%. However, use of the correction procedure described below will reduce this error to 3.6% over the same concentration range. This correction procedure involves the measurement of the isotopic abundances, a_1, a_2, b_1, and b_2, but only with an accuracy of $\pm 2\%$ for this degree of improvement.

The correction of calibration plots to a theoretically more nearly linear form can be achieved by the following procedure. When a_2A cannot be neglected we may approximate R by a quadratic expression so that

$$R = k_1A^2 + k_2A + k_3$$

A least squares procedure[23] may now be used to find the best values for the constants k_1, k_2, and k_3 from the calibration values for R and A. Then, unknown concentrations may be determined from measured ratios using a straight line fitted by the least squares method to a plot of A against $(R - k_1A^2)$. As the correction term k_1A^2 is small, A can be expressed in terms of the measured ratio R and the known abundances a_1, a_2, b_1, and b_2 as

$$A = \frac{B(b_2R - b_1)}{(a_1 - a_2R)}$$

Thus, in practice, the calibration plot used is one of

$$A \text{ against } R - k_1\left[\frac{B(b_2R - b_1)}{(a_1 - a_2R)}\right]^2$$

Further reduction of the error due to non-linearity should be possible, for example by weighting the calculated values of $(R - k_1A^2)$ used to give the straight-line plot[24].

However, greater accuracy of measurement of the abundances a_1, a_2, b_1, and b_2 would be required to take advantage of such improvements.

(ii) Interference from the standard at mass M_1, *i.e.* b_1B is not negligible compared with a_1A in eqn. 1. In this case, as b_1B increases relative to a_1A, the accuracy with which the sample level can be determined by measurement at M_1 decreases. In the testosterone determination described in this paper, the contribution of the standard at M_1 is approximately twice that of the smallest sample at the same mass. The limit for this ratio of standard to sample contribution at M_1 depends on a number of factors, including the sample levels employed, but a value of two represents a reasonable practical limit for this type of determination. The maximum permissible amount of internal standard defined in this way should then be employed in any experiment where the concentration range over which quantitative determination is possible, recognising the limitations outlined in (i), is to be a maximum also.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Mrs. E. Isles for help with the preparation and typing of the manuscript.

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