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THE MEASUREMENT BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF OESTRA-1,3,5,-TRIENE-3,15α,16α,17β-TETROL (OESTETROL) IN PREGNANCY URINE

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SUMMARY

A method is reported for the rapid quantitative determination of oestra-1,3,5 (10)-triene-3,15α,16α,17β-tetrol (oestetrol) in pregnancy urine. The technique uses a gas chromatograph coupled to a mass spectrometer detector. The mass spectrometer is tuned to monitor the *m/e* 191 ion produced during the fragmentation of oestetrol tetra(trimethylsilyl) ether by electron bombardment. The signal from the oestetrol is compared with the signal from a known mass of a synthetic standard, 4-methyl oestra-1,3,5(10)-triene-1,15α,16α,17β-tetrol, which fragments similarly to oestetrol. The synthetic standard is added to a hydrolysed urine sample before extraction to correct for losses during extraction. Because of the specificity of this technique, no purification is necessary other than the extraction of phenolic steroids. The precision is 6% and a linear response of the detector is observed over the range of oestetrol concentrations normally met. The technique is sensitive enough to allow measurement of oestetrol from the tenth week of pregnancy. One nanogram of oestetrol can be detected with a signal-to-noise ratio of 6:1 and the limit of measurement is five nanograms of oestetrol in a sample injected into the gas chromatograph.

INTRODUCTION

The levels of oestra-1,3,5-triene-3,15α,16α,17β-tetrol (oestetrol) in pregnancy urine are of interest because this steroid has been shown to be predominantly foetal in origin[1,2] and consequently the levels may reflect the stage of foetal development. Although oestriol can be measured in unpurified pregnancy urine extracts by gas chromatography (GC) with conventional detectors, oestetrol cannot be measured satisfactorily by such methods because of the lower concentration of this steroid.

GC methods for oestetrol measurement published so far[3,4] incorporate liquid phase chromatography. This is necessary with flame ionisation detectors to obtain sufficient specificity and adequate sensitivity. In the present GC method, no liquid phase chromatography stages are used. Specificity is provided by the use of a mass spectrometer (MS) as a highly specific detector for the gas chromatograph. This

technique allows measurement of ng quantities of oestetrol in unpurified extracts of phenolic compounds from pregnancy urine.

When a mass spectrometer is tuned to monitor one mass-to-charge ratio and, as such, is used as a selective detector for a gas chromatograph, high detector sensitivities can be obtained. These sensitivities are of a much higher order than the normal sensitivity of a gas chromatograph–mass spectrometer (GC–MS) combination when it is used for qualitative analysis. This increase is obtained by using wider slit widths, lower band width amplifiers and the best ion source parameters and magnet position for the ion under study. Moreover, in addition to its high sensitivity, the mass spectrometric detector also possesses an intrinsically high selectivity. When this selectivity of the mass spectrometer is coupled with the separating efficiency of GC, the discriminating ability is such that impure samples can be analysed for specific components in the presence of hundredfold excesses of closely related compounds.

Such ion-specific analysis can suitably be applied to the measurement of oestetrol because the mass spectrum of the tetra(trimethylsilyl) ether derivative of oestetrol has a strong peak at *m/e* 191 (ref. 5).

Although combined GC–MS, when used in the ion-specific mode, is a sensitive and selective technique, the problem is the quantitation, or more specifically, the correlation of the signal generated with the mass of a particular compound injected into the gas chromatograph. Apart from the normal losses or uncertainty in the injection of liquid samples into the gas chromatograph and the possible losses of polar compounds on the column, the mass spectrometric detector introduces varying signal-to-mass response for every varying voltage or current which influences the intensity, focussing or measurement of the ion beam. Although such factors, which could affect the signal to mass response, are stabilised as far as possible, absolute measurements based on signal-to-mass ratio are unreliable and cannot at present form a basis for precise measurement. However, comparison of the mass spectrometer signal from two nearly identical compounds eluting from a gas chromatography column close to each other, should be a reliable method of measuring the mass of one of them if the mass of the other is known, for in such a case the only variations in detector sensitivity which will affect the result are those occurring between the elution of the two peaks. This approach gives excellent results in electron capture detection[6], but in the case of the specific ion detector the requirements for the internal standard are more stringent: the standard must possess the same signal generating group as the compound to be measured, yet differ in some way which allows gas chromatographic separation. This problem is complicated because the signal generating group in this connection is often poorly defined, because competing fragmentations (possibly originating many carbon atoms distant) involving the signal generating group, will influence the signal-to-mass response. In this measurement of oestetrol a reference compound (4-methyloestra-1,3,5(10)triene-1,15α,16α,17β-tetrol) was synthesised; this compound has the same B, C and D ring configuration as oestetrol and has a phenolic hydroxyl group in the "A" ring but the A ring is rearranged. Because the local environment is the same during the origin of the *m/e* 191 ion in both the natural and reference compounds, the signal per mole response should be the same in each case.

MATERIALS, METHODS AND INSTRUMENTATION

Materials

Ethyl acetate, benzene and ether were analytical grade and fresh but otherwise unpurified. Bis(trimethylsilyl) acetamide and trimethylchlorosilane (Pierce Chemical Company) were redistilled and stored in sealed ampoules before use. The synthetic oestetrol was prepared from 1-hydroxy-4-methyloestra-1,3,5(10)trien-17-one which was prepared by acetolysis (dienone–phenol rearrangement) of androsta-1,4-diene-3,17-dione (ref. 7). The Δ^{15} unsaturation was introduced by a standard method[8] and the Δ^{15}-17-ketone was reduced and hydroxylated according to the method of FISHMAN AND GUZIK[9,10]. The tetrol was purified by thin-layer chromatography (TLC) and crystallised from aqueous methanol, m.p. 153–155°. The orientation of the 15 and 16 hydroxyls in the tetrol isolated was inferred from the fact that it was the major product of the hydroxylation, and the more polar of the two tetrols formed. The structure was confirmed by mass spectrometry. A standard solution of the synthetic oestetrol was prepared in 50% aqueous ethanol of a concentration equal to approximately 20 μg per ml and this solution was stored at 0° in $\frac{1}{2}$ ml sealed glass ampoules which were opened as required. Authentic crystalline oestetrol was obtained as a gift from Professor S. SOLOMON.

Methods and instrumentation

Hydrolysis and extraction of urine. Aliquots of urine (5 ml) were diluted to 10 ml with 0.1 *N* acetate buffer and 50 μl *Helix pomatia* extract (5,000 Roy units sulphatase, 40,000 Fishman units glucuronidase) were added. The mixture was incubated for 24 h at 37°, then a further 50 μl of *Helix Pomatia* extract were added and the incubation was repeated. 25 μl of the synthetic standard solution was added to the hydrolysed urine which was saturated with salt and extracted with 20 ml ether–ethyl acetate (2:1), the organic extract was washed with 5 ml saturated carbonate solution pH 10.5 (ref. 11), then washed with 20 ml 0.5 *N* sodium hydroxide. The alkaline extract was removed, acidified, saturated with sodium chloride and re-extracted with 20 ml ether–ethyl acetate. The organic layer was washed with 5 ml saturated sodium bicarbonate solution, further saturated with sodium chloride, dried with magnesium sulphate and evaporated to dryness on a rotary evaporator. The residue was dissolved in 250 μl of ethanol and 50 μl aliquots were taken for analysis, these were evaporated under vacuum at the bottom of a 50 mm long tube of 2 mm I.D. 20 μl of a 20% solution of bis(trimethylsilyl) acetamide in benzene and 5 μl of a 20% solution of trimethyl chlorosilane were added and the tube was sealed in a flame. The tubes were broken open for analysis after a minimum of 2 h and 2–3 μl aliquots were injected into the GC–MS combination, allowing most of the solvent to be sucked through a capillary in the outlet of the chromatograph. Alternatively, the accelerating voltage was dropped to 2 kV during the emergence of the solvent.

Gas chromatograph–mass spectrometer. The gas chromatograph was a Hewlett Packard Model 402 equipped with U shaped columns. Helium was used as carrier gas. The columns were 5 ft. long, 2 mm I.D., packed with $\frac{1}{2}$% OV-1 on 100/120 mesh "Gas-Chrom Q" with a resolution of 400 plates per foot, columns were fitted between an injection mount and an auxillary detector position, the fitting at this position has inserted through it a stainless-steel capillary through which the effluent can be sucked

(by an auxillary diaphragm pump) during the emergence of the solvent. The effluent from the gas chromatograph is transferred via a Swagelok coupling to a resistively heated metal capillary leading to a WATSON–BIEMAN separator[12]. The separator was silanised *in situ* using dimethyl diacetoxysilane in the vapour phase. The enriched sample from the separator is fed through a glass line into an Associated Electrical Industries MS 12 mass spectrometer mounted on vibration-free concrete. Typical conditions during a specific ion analysis were as follows:

Temperature: gas chromatograph oven 250°, gas chromatograph detector block 250°, steel transfer capillary 240°, molecular separator 240°, re-entrant tube and glass lines 230°, source block 230°.

Pressures: 50 lb./sq. in. helium pressure at column inlet, 20 lb./sq. in. at column outlet, 1 Torr at outside of glass frit, 1×10^{-5} Torr in source housing and 1×10^{-7} Torr in analyser.

Voltage and currents: Accelerating voltage 8 kV, trap current 100 μA, ionising voltage 20 eV, electron multiplier voltage 1.8 kV. The source slit width was 0.010 in., analyser slit width was 0.015 in. Magnet current was allowed to stabilise over a period of about 1 h prior to ion specific analysis. Magnet current was adjusted (with the help of a mass marker attachment) to give maximum signal on the collector meter from the small peak at *m/e* 191, present in the mass spectrum of the bleed from the OV-1 column. The signal output was then switched to the recorder ready for ion specific analysis.

The mass spectrometer head amplifier was modified to incorporate a 1000 MΩ resistance in the grid leak of the first valve; this allows lower electron multiplier voltages to be used. Signal passed to the main signal amplifier through the bandwidth filter set at 1 c.p.s. and the output from the signal amplifier was fed through a 10:1 voltage divider network and a further resistance capacitance filter to a pen recorder (Rikadenki Model B341X-A) with input selection at 100 mV. The output was fed simultaneously through a 100:1 voltage divider to a "Chromalog 2" electronic integrator (Kent instruments), set for 1 V maximum input. Signal from the total ion current monitor was fed to a second channel of the pen recorder. Areas under peaks were determined either from peak height measurements or from the integral values from the electronic integrator.

RESULTS AND DISCUSSION

Hydrolysis

Enzyme hydrolysis was used because acid hydrolysis sometimes gave appreciable concentrations of epioestriol as an artifact and although epioestriol gives only a weak 191 ion at 20 eV, the peak overlaps that of the synthetic oestetrol. Enzyme hydrolysis was carried out for 48 h, but reducing this time to 24 h results in very little loss of accuracy.

Instrument stability

The use of a mass spectrometer to monitor one mass-to-charge ratio requires of the instrument a high stability. The particular instrumentation used in this study was sufficiently stable to allow specific ion analysis at high sensitivity throughout a working day, with the exception of 1 h at the start during which the source supplies

settle and after which final tuning of the ion beam is carried out. During a period of specific ion analysis the magnet current is left continuously at the required value and no change is necessary from day to day.

Suitability of internal standard

The suitability of the internal standard has to be considered from the aspect of the correction for extraction losses and the quantitation in the final measurement. During the extraction of a urine, the synthetic oestetrol does not behave identically to the natural oestetrol. The relative behaviour of the synthetic and natural steroid was studied by incomplete extraction followed by re-extraction at various stages in the normal procedure. During extraction with sodium hydroxide the ratio of synthetic to natural in the re-extracted sample was the same as in the original extraction, indicating that losses at this stage are fully compensated for. However, in the ether–ethyl acetate extractions there is preferential extraction of the synthetic tetrol and thus if the recoveries are not high some innaccuracy will be introduced at these stages. The overall recovery of oestetrol is 70% and consequently the preferential extraction of the synthetic standard will introduce some error, although all accidental spillages and losses during the alkali extraction stage will be allowed for.

The recovery could be improved by using repeated extractions at each stage but the accuracy using the present procedure is sufficient for the purpose of the analysis. Moreover, because of the very high polarity of the oestetrol the exhaustive extractions necessary to get high recoveries would add more contamination to the extract.

The requirement of the internal standard during the mass spectral measurement is that it should give the same ion current per mole as does oestetrol. From a theoretical consideration, the standard used should nearly satisfy this condition. It is not certain by exactly which mechanism the *m/e* 191 ion ($C_7H_{19}O_2Si_2$) is produced, but it is probable that it arises through silyl migration and a ring enlarged intermediate[13]. In any case the origin of the ion at 20 eV is confined to the D ring. The production of the 191 ion could only be affected by long range effects or by prior ionisation elsewhere in the molecule. The long range effects are unlikely, due to the number of intervening carbon atoms and prior ionisation is kept to a minimum by using a low ionising voltage. The mass spectra of oestetrol and synthetic oestetrol are shown in Fig. 1. The signal to mass responses of the oestetrol and synthetic oestetrol are very similar, the ratio being 1.0 ± 0.1. The variation is long term and depends on the instrumental conditions. Because of this long term variation, standard mixtures are analysed every day.

Theoretically the Bieman separator will discriminate between the natural and synthetic oestetrols in favour of the higher molecular weight synthetic standard. In practice, since the molecular weight difference is small and the effect is partly offset by the theoretical increase in the signal per mole response of the lower molecular weight compound, no such effect is observed.

Precision

The precision of this application of ion specific analysis to the measurement of oestetrol in pregnancy urine is represented by a standard deviation of 6% (36 paired samples); this figure was obtained by duplicate determinations of steroid levels in

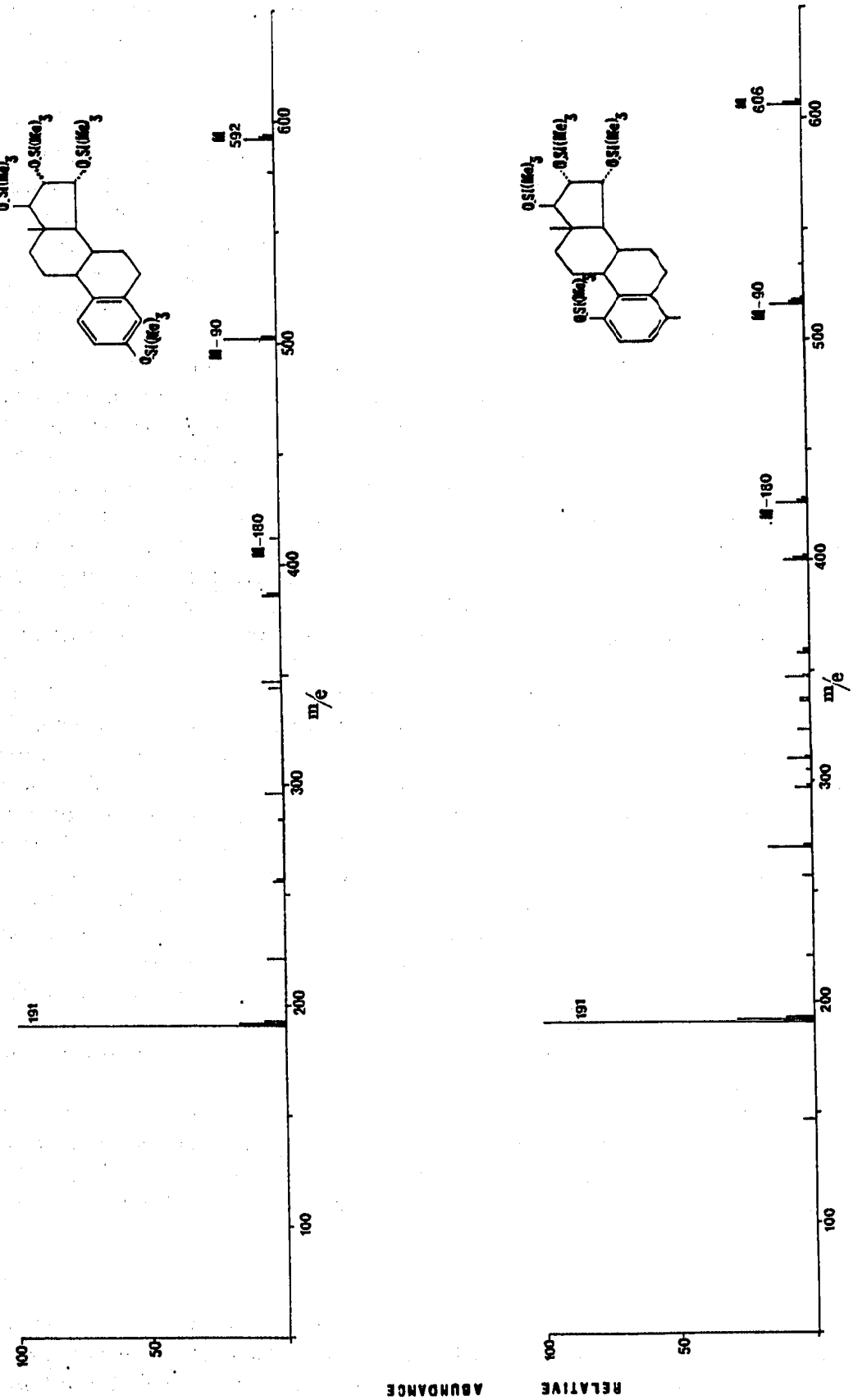

Fig. 1. Mass spectra of 3,15*a*,16*a*,17*β*-tetrahydroxyoestra-1,3,5(10)-triene tetra(trimethylsilyl) ether and 4-methyl-1,15*a*,16*a*,17*β*-tetrahydroxyoestra-1,3,5(10)-triene tetra(trimethylsilyl) ether. Energy of bombarding electrons, 20 eV.

urine samples by the method of SNEDECOR[14]. This precision value represents not only the variation in the final analytical determination but also the variation due to losses in extraction. The variation in the ion specific analysis of a urine extract is represented by the precision of duplicate determinations of the oestetrol to synthetic oestetrol ratio in the same extract samples; this gives a value of the standard deviation of 3% (38 samples). The difference in these observed standard deviations gives some idea of the imprecision introduced by the extraction technique.

Using the technique presented here the ion specific analysis of a urine sample is less precise than the similar analysis of pure standards; one reason for this is the difficulty in getting accurate area measurements from the relatively complicated traces of the urine samples. This difficulty could be overcome by adding a further purification stage, but because the internal standard could not be used throughout the procedure, the overall precision would in fact be lower. The precision of the ion specific analysis for pure samples using electronic integration for quantitation is 2.5% (21 samples). This more closely represents the precision of the actual measuring technique and might be more nearly attained in samples where more readily extractable and more easily recovered compounds are to be measured.

Specificity

The conditions which must be met before a compound can interfere with the measurement of oestetrol are that the compound must have both a relatively strong peak at *m/e* 191 and also an elution volume nearly equal to either oestetrol or the synthetic internal standard. No compound has yet satisfied these criteria fully. The

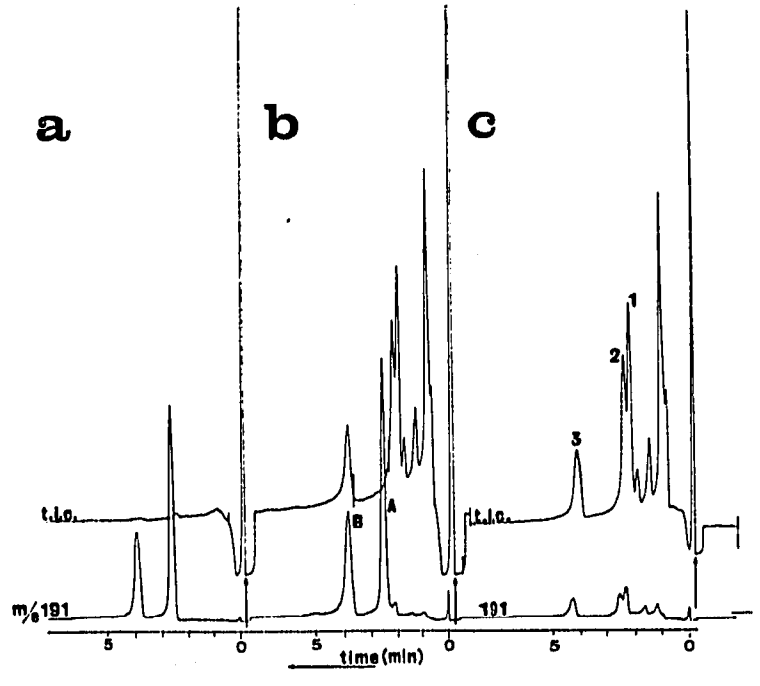

Fig. 2. *m/e* 191 specific trace (lower) and total ion current trace (upper) of standard mixtures of steroids. (c) Mixtures of steroid silyl ethers. Peak 1 = oestriol, peak 2 = 17-epioestriol, peak 3 = cholesterol (2 μg of each component). (b) Mixture of steroids as in (c) but 1 μg of each component with 20 ng synthetic oestetrol (A) and 10 ng of natural oestetrol (B); t.i.c. monitor sensitivity 2 × that in (c). (a) Mixture of oestetrols alone. Conditions as in text.

specificity is enhanced by working with a low ionising voltage; dropping the ionising voltage from 25 eV to 20 eV results in only a small decrease in the sensitivity of the detector to oestetrol and the standard, but greatly reduces the sensitivity of the detector to other compounds. Monitoring the 191 ion at 20 eV the signal to mass response is 10,000 times greater for oestetrol than for oestriol. The relative responses of the 191 specific trace and the total ion current (t.i.c.) monitor are demonstrated (Fig. 2) for the synthetic and natural oestetrol and for one hundred times the weight of various C_{18} and other steroids. The trace b shows that the ng amounts of oestetrol are masked by the other compounds almost completely on the total ion current trace, but are readily measurable on the selective 191 trace. Thus a further element of specificity is introduced if the ratio of the response from the selective detector to the response from the t.i.c. detector is considered.

Accuracy

The accuracy is largely dependant on the relative behaviour of oestetrol and the internal standard. As has been mentioned, the ratio of response of the selective detector to the two is 1.0 $\pm$ 0.1. This ratio was checked during ± 2 V variations in ionising voltage, $\pm 5\%$ variations in the photomultiplier voltage (compensating for the overall sensitivity change by altering recorder gain) and during small variations in ion repeller voltage (again keeping overall sensitivity constant by altering recorder gain). No significant change in the ratio was observed. It is probable, however, that the small changes in the ratio that do occur are partly due to chromatographic conditions. Partial loss due to catalytic activity and adsorbtion will be experienced by both the natural oestetrol and the synthetic, but, because the natural oestetrol has an appreciably longer retention time, greater losses occur for this compound. Certainly with small samples of oestetrol (less than five ng) the ratio of synthetic to natural becomes unreliable and it is reasonable to assume that losses at this level are becoming significant.

The accuracy is connected with the specificity and if the natural and synthetic oestetrols are not separated from interfering material, accuracy will suffer. There is no evidence for heterogeneity of the peaks and mass spectra at various points in the peaks indicates homogeneity. Fig. 3 shows a typical 32 week pregnancy urine with and without the internal standard showing that there is no peak masked by the standard. To check whether any peak is masked by the oestetrol, an extract of pregnancy urine was run on TLC; the area corresponding to oestetrol was scraped off. The remainder was eluted and analysed by ion specific analysis. No significant peak was present at the oestetrol position.

The linearity of response of the mass spectrometric detector was studied over the range of oestetrol concentrations met during analysis of normal pregnancy urines. Different volumes (10–250 μl) of a standard solution of crystalline oestetrol (16.9 μg per ml) were added to 5 ml aliquots of male urine which contained no oestetrol. The concentrations of the samples were equivalent to urines of 34 μg–1 mg oestetrol per litre. Twenty-two samples were individually extracted and each was measured in duplicate. The urines were processed using the normal procedure. The ratio of the signal per mole response of synthetic to natural oestetrol in a standard mixture run on the same days as the analysis was 1.00 and using this value a linear plot (Fig. 4) is obtained with a correlation coefficient of 0.997. This demonstrates the linearity of

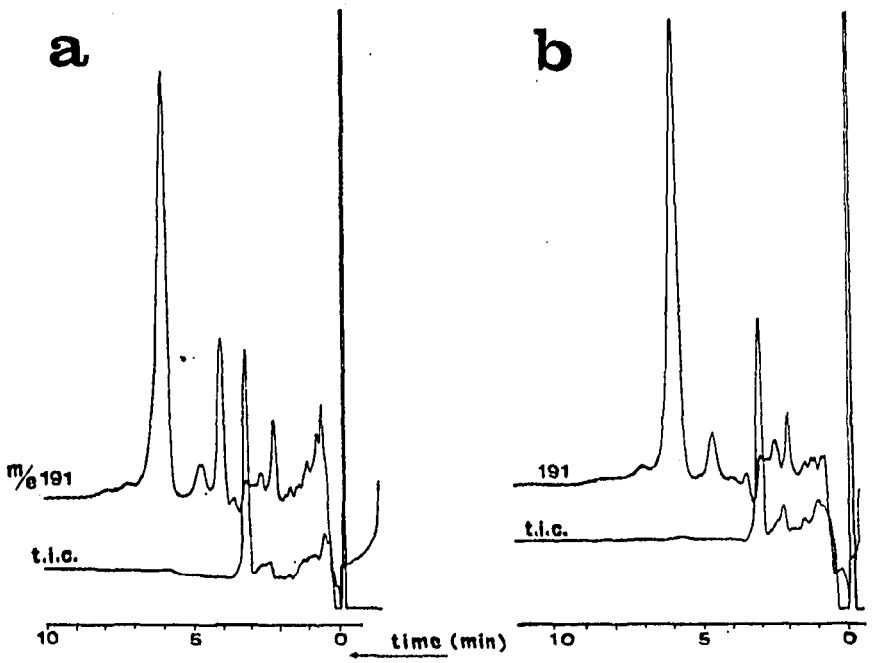

Fig. 3. (a) Silylated extract of 32 week pregnancy urine with synthetic oestetrol added; (b) as for (a) but no synthetic oestetrol added. The large peak on the t.i.c. trace is oestriol trimethylsilyl ether.

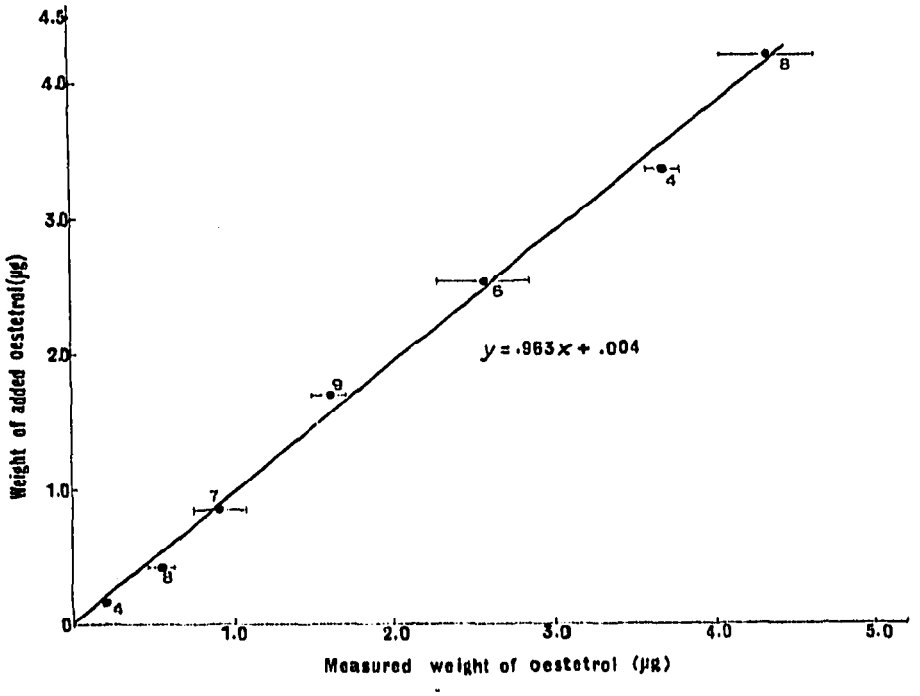

Fig. 4. Plot of added oestetrol against amount measured, linear regression analysis gives amount added (μg) as 0.963 times amount measured plus 0.004 μg. Numbers of samples are given beside the points.

the detector over the range used. Pregnancy urines can be kept within this range by using 1.0 ml aliquots of late pregnancy urine, this brings a further advantage, that the urine can be diluted tenfold with buffer to minimise the effects of enzyme inhibitors.

Sensitivity

The limit of determination is five ng per sample. Below this value the standard deviation begins to rise, presumably due to losses on the column, molecular separator and inlet lines. One ng of oestetrol can readily be detected with a signal to noise ratio of 6:1.

The sensitivity limit in the determination of oestetrol which enters the ionisation chamber is set by the bleed from the silicon polymer stationary phase. There is a significant bleed peak at *m/e* 191 which helps in tuning in the instrument to peak 191 but although the constant component of the signal from the bleed can be compensated for electrically, the noise which is introduced limits the sensitivity of the detector. The sensitivity is sufficient to measure oestetrol levels in urine samples from the 24th week of pregnancy. Oestetrol levels from the 10th week of pregnancy can be measured by using larger aliquots of urine.

Practicability

The selectivity of the specific ion measurement allows the purification of samples to be cut to a minimum. The extraction of the phenolic steroids and the formation of silyl ethers are all that is necessary. A technician can extract 12 samples at a time and can take 24 samples (12 in duplicate) a day through the extraction and silylation procedure. The limitation on the speed of measurement of the silylated extracts is the retention time of oestetrol trimethylsilyl ether. This retention time can be cut to 5 min, and under these conditions upwards of 40 samples (20 in duplicate) can be analysed in one day.

Conclusion

The high specificity of the mass spectrometer as a detector allows the measurement of small amounts of oestetrol in relatively impure pregnancy urine extracts. The high specificity is accompanied by a high sensitivity and the precision of the technique is moderate. Both sensitivity and precision could be greatly improved with instrumental advances.

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