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# Determination of Oestradiol-17β in Human Serum by Isotope Dilution-Mass Spectrometry

## Definitive Methods in Clinical Chemistry, II

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**Summary:** A definitive method is described for the measurement of oestradiol-17β in human serum. The method is based on the principle of isotope dilution-mass spectrometry. The analytical procedure consists of the following steps:

(1) addition of [4-$^{14}$C]oestradiol-17β to the plasma sample;
(2) extraction of the $^{14}$C-labelled and the non-labelled oestradiol-17β with dichloromethane;
(3) purification of the oestradiol-17β fraction by column chromatography on Sephadex LH-20;
(4) formation of the 3,17β-diheptafluorobutyric ester of oestradiol-17β;
(5) selected ion monitoring at m/z-values 664 and 666 by gas liquid chromatography-mass spectrometry.

The accuracy of the method is based on the high specificity of mass spectrometry and on the exact control of recovery employing the principle of isotope dilution. Coefficients of variation from day to day varied between 1.0% and 2.5% in the range of 0.319 to 3.53 nmol/l. The lower limit of detection (ratio of signal to noise 3:1) is 5 pg oestradiol-17β per sample.

*Bestimmung von Östradiol-17β im menschlichen Serum mit Hilfe der massenspektrometrischen Isotopenverdünnungsanalyse*

*Definitive Methoden in der Klinischen Chemie, II*

**Zusammenfassung:** Es wird eine definitive Methode zur Bestimmung von Östradiol-17β im menschlichen Serum beschrieben, die auf dem Prinzip der massenspektrometrischen Isotopenverdünnungsanalyse beruht. Das analytische Verfahren umfaßt folgende Schritte:

(1) Zugabe von [4-$^{14}$C]Östradiol-17β zu der Serumprobe;
(2) Extraktion des $^{14}$C-markierten und des nachzuweisenden nichtmarkierten Östradiol-17β mit Dichlormethan;
(3) Reinigung der Östradiol-17β-Fraktion durch Säulenchromatographie an Sephadex LH-20;
(4) Bildung des 3,17β-Diheptafluorbuttersäureesters von Östradiol-17β;
(5) massenspektrometrische Registrierung ausgewählter Ionen bei den m/z-Werten 664 und 666 während der Gaschromatographie.

Die Richtigkeit der Methode beruht auf der hohen Spezifität des massenspektrometrischen Nachweises und auf der genauen Kontrolle der Wiederfindung unter Anwendung des Isotopenverdünnungsprinzips. Die Variationskoeffizienten von Tag zu Tag lagen zwischen 1,0% und 2,5% im Bereich von 0,319 und 5,53 nmol Östradiol-17β/l. Die untere Nachweisgrenze (bei einem Signal-zu-Rausch-Verhältnis von 3:1) ist 5 pg Östradiol-17β pro Probe.

## Introduction

At present, methods for the determination of steroid hormones in human serum are based almost entirely on the principle of enzyme or radioimmunoassay. These methods are widely used and have proved to be of great value in the diagnosis of endocrine disorders, even though the results obtained by different methods and laboratories seem to vary greatly, as recently shown by a report on the results of quality control surveys for the determination of steroid hormones (1). It therefore appeared necessary to develop reference and definitive methods for the determination of steroid hormones, according to the recommendations on quality control of the International Federation of Clinical Chemistry (2, 3), in order to provide a basis for the improvement of the accuracy of routine methods.

In a previous article in this series (4) a definitive method for the measurement of cortisol in human blood was reported. In the present paper a definitive method for the determination of oestradiol-17β involving isotope dilution-mass spectrometry is proposed.

## Principle of the Method

Oestradiol-17β in a serum sample is equilibrated with a definite amount of isotopically labelled oestradiol-17β. The steroids are extracted by using an organic solvent and then subjected to column chromatography for further purification. For gas chromatography-mass spectrometry, the phenolic steroids are converted to heptafluorobutyric ester derivatives. This is followed by combined gas chromatography-mass spectrometry: During gas chromatography the molecular ions of the non-labelled as well as of the isotopically labelled oestradiol-17β derivative are recorded continously by the mass spectrometer. The concentration of oestradiol-17β in the serum sample is calculated from the isotope ratio as measured by mass spectrometry and from the known amount of isotopically labelled oestradiol-17β added to the serum sample.

## Materials and Procedure

### Reagents

[4-$^{14}$C]Oestradiol-17β (spec. radioactivity 1.924 GBq/mmol, 84% $^{14}$C) was obtained from the Radiochemical Centre, Amersham, UK; the radiochemical purity of the material was checked by thin layer chromatography on plates of silanised silica gel (Merck, Darmstadt, FR Germany); the radioactivity was monitored by using the LB 282 TLC Linear Analyzer (Berthold, Wildbad, FR Germany). Oestradiol-17β was a reference preparation obtained from the Tenovus Institute for Cancer Research, Cardiff; the same material, crystallised from aqueous ethanol, was used as preliminary reference material (017/3/6/137/78/BCRUK) for a project of the Community Bureau of Reference of the Commission of the European Communities. Heptafluorobutyric anhydride was purchased from Merck (Darmstadt, FR Germany).

Oestriol-3,16α,17β-pentafluoropropionic ester, used as reference substance for calibrating the mass spectrometer, was prepared by reacting oestriol (Schering, Berlin-West) with a mixture (1:1 by vol) of pentafluoropropionic anhydride (Pierce, Rotterdam, The Netherlands) and dry acetonitrile (Merck, Darmstadt, FR Germany) for 30 min at 60 °C. Dichloromethane, used for the extraction of oestradiol-17β from the serum samples, was distilled prior to use. Sephadex LH-20 for column chromatography was obtained from Pharmacia (Freiburg, FR Germany) and α-naphthol was supplied by Merck (Darmstadt, FR Germany). All reagents and solvents were of analytical grade.

### Glassware

Volumetric flasks used for the preparation of the oestradiol-17β standard solutions (concentrated stock solution and diluted solution for final use) were officially calibrated. For the dilution of the oestradiol-17β standard stock solution as well as for the sampling of serum, enzyme test pipettes (1 ml, 2 ml and 5 ml total volume, Blaubrand-Ionodur, Brand, Wertheim, FR Germany) were calibrated prior to use by pipetting and weighing appropriate amounts of water at 20 °C.

For pipetting the final diluted oestradiol-17β standard solution and the [4-$^{14}$C]oestradiol-17β standard solution a 100 µl and a 25 µl syringe (SGE, Melbourne, Australia) equipped with repeating adaptors were used respectively. The 100 µl syringe was calibrated by sampling and weighing 50 µl of water. By using syringes with direct displacement of the volume the differences of surface tension between water and ethanol do not have any effect on the calibration. The loss of water, due to evaporation of the solvent, during the sampling and weighing procedure, was corrected by weighing the water samples 45 s and 90 s after sampling and calculating the original weight of 0 s.

### Weighing Procedure

The reference material and the 50 µl water samples for calibrating the 100 µl syringe were weighed with an electronic balance (model 4135, Sartorius, Göttingen, FR Germany). The accuracy of the balance was checked with officially calibrated weights.

### Instruments and Settings

An LKB 9000 combined gas chromatograph/mass spectrometer equipped with the LKB 2031 computer system (Boehringer, Mannheim, FR Germany) or a Finnigan 1020 system (Finnigan MAT, Bremen, FR Germany) were used alternately. With the LKB instrument, the ion source as well as the molecular separator were operated at 290 °C. The energy of the electron beam was 26 eV and the ionising current 120 µA. The high voltage at the electron multiplier was 3400 V. The multiplier of the Finnigan instrument was set to 2200 V. Both instruments were operated at a resolution of about 500 (10% valley definition). For gas liquid chromatography a fused silica capillary column (0.32 mm × 25 m) was used containing a film of 0.1 µm SE-30 or SE-52 which is chemically bonded to the surface (Macherey-Nagel, Düren, FR Germany). The column was operated at 260 °C using helium as a carrier gas (inlet pressure 40 kPa). Samples were introduced into the column by a moving-needle-solid-inlet system (5) obtained from Chrompack (Berlin-West).

### Sample preparation

Twenty five microlitres of an ethanolic solution, containing approximately 280 pg of [4-$^{14}$C]oestradiol-17β, were added to 0.5–5 ml serum, using the 25 µl syringe equipped with the repeating adaptor. In order to obtain 1:1 isotope ratios, which are most favourable in definitive measurements, the amount of serum to be used in the experiment was first determined by a preliminary analysis. The serum sample was equilibrated with the labelled oestradiol-17β by gently shaking the test tube for 30 min at room temperature. Dichloromethane (15 ml), containing 2 µg α-naphthol as a carrier substance, was added and the mixture mechanically shaken for 30 min at room temperature.

The aqueous phase was then drawn off, and the organic phase washed twice with 1 ml water and evaporated to dryness under nitrogen at 60 °C.

### Column Chromatography

The column was prepared by filling a glass column (30 × 0.4 cm), equipped with a fritted disc, with 1 g of Sephadex LH-20. The column was rinsed with 100 ml of methanol and then equilibrated with 15 ml of a solvent mixture composed of dichloromethane/cyclohexane/methanol/water (100 + 80 + 15 + 1, by vol.). The residue of the purified plasma extract was taken up twice with 100 µl of the solvent and transferred to the column. The column was first washed with 9 ml of the solvent mixture and the oestradiol-17β fraction eluted with an additional 2 ml of solvent. The elution pattern is dependent on temperature, and there may be slight variations between batches of Sephadex LH-20. It is therefore advisable to check the exact position of the oestradiol-17β fraction by chromatographing a small sample of [4-$^{14}$C]oestradiol-17β on a separate column and measuring the radioactivity in 0.5 ml fractions in a liquid scintillation counter (Nuclear Chicago Mark II, Zinsser, Frankfurt, FR Germany). A typical elution profile is shown in figure 1. The oestradiol-17β fraction obtained after column chromatography was collected in conical test tubes (total volume 2 ml) and the organic phase evaporated under nitrogen at 60 °C.

When the columns have been washed after each chromatographic run, they may be reused as many as ten times, before it is necessary to repack them.

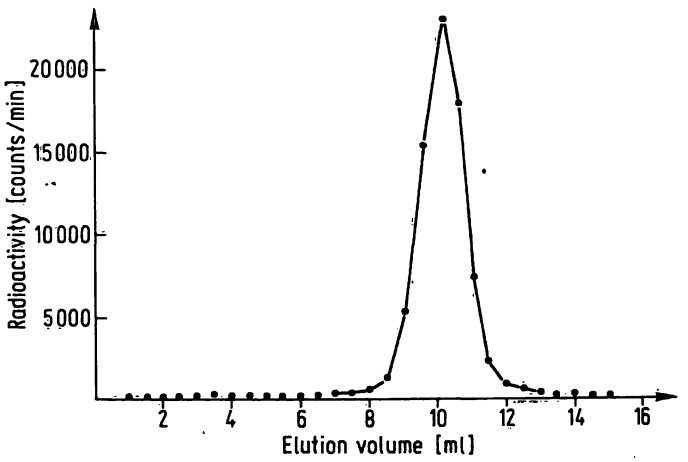


Fig. 1. Elution profile after chromatography of 1.85 KBq [4-$^{14}$C]oestradiol-17β on a column (30 × 0.4 cm) of Sephadex LH-20 (1 g).

### Formation of Derivatives

The oestradiol-17β containing residue in the conical tube was reacted with a mixture of heptafluorobutyric anhydride and acetonitrile (1 + 4, by vol.) for 30 min at 60 °C. The reagent mixture was evaporated under nitrogen at 60 °C.

### Preparation of Standard Solutions

For the measurement of standard samples, calibrated solutions of $^{14}$C-labelled and non-labelled oestradiol-17β were prepared. For the $^{14}$C-labelled oestradiol-17β standard solution the radioactively labelled oestradiol-17β (370 KBq) which is purchased in 0.5 ml benzene/ethanol was transferred to a test tube (2 × 8 cm); the solvent was evaporated and the [4-$^{14}$C]oestradiol-17β dissolved in 10 ml ethanol, thus yielding a concentrated stock solution. Fifty microlitres of this solution were diluted with 25 ml ethanol, which produces a solution of approximately 280 pg/25 µl. The solutions were stored at −20 °C and allowed to equilibrate to 20 °C prior to use.

For the preparation of the non-labelled oestradiol-17β standard solution, an exact amount of 2.284 mg oestradiol-17β was weighed into a 500 ml volumetric flask (officially calibrated, estimated inaccuracy 0.25 ml). The flask was filled up to the calibration mark with methanol at 20 °C and the contents shaken vigorously in order to bring about complete dissolution and distribution. This solution was used as concentrated stock solution and stored at −20 °C.

For the preparation of a diluted oestradiol-17β solution, 0.5 ml of the concentrated stock solution was transferred to a 500 ml volumetric flask (officially calibrated, estimated inaccuracy less than 0.25 ml) using an enzyme test pipette (calibrated by pipetting and weighing 12 samples of 0.5 ml water at 20 °C, volume = 0.50147 ml ± 0.0016 ml (SD)). The 500 ml volumetric flask was filled with methanol at 20 °C to the calibration mark and the contents shaken vigorously for distribution. The final diluted oestradiol-17β standard solution contained 0.22907 ng/50 µl. This solution was stored at −20 °C prior to use.

### Preparation of Standards

Three different standards were included in each analytical series:

Standard *1*, prepared in triplicate, consisted of a mixture of 100 µl of the ethanolic [$^{14}$C]oestradiol-17β standard solution (pipetted with the 25 µl syringe equipped with a repeating adaptor) and 150 µl of the diluted oestradiol-17β standard solution. The non-labelled oestradiol-17β solution was sampled by pipetting 50 µl thrice with the calibrated 100 µl syringe which was fixed with the repeating adaptor at the 50 µl mark. The exact volume of the syringe was determined by pipetting and weighing 50 µl water twelve times at 20 °C; the corrected volume was 49.4496 µl ± 0.1287 µl (SD). With respect to this calibration, the exact amount of non-labelled oestradiol-17β in standard sample *1* was calculated to be 0.6796 ng.

Standard *2*, prepared in six replicates, consisted of a mixture of 100 µl of the ethanolic [4-$^{14}$C]oestradiol-17β standard solution and 200 µl of the diluted oestradiol-17β standard solution. For sampling the non-labelled oestradiol-17β solution, 50 µl were pipetted four times with the calibrated 100 µl syringe. The amount of non-labelled oestradiol-17β in standard *2* was 0.9062 ng.

Standard *3*, prepared in triplicate, was a mixture of 100 µl of the ethanolic [4-$^{14}$C]oestradiol-17β standard solution; the non-labelled material was pipetted with the calibrated 100 µl syringe (50 µl five times). Standard *3* contained 1.1328 ng oestradiol-17β.

The amount of [4-$^{14}$C]oestradiol-17β in the standard samples was four times the quantity added to the serum samples. Therefore, in the calculation of analytical results, the amounts of non-labelled oestradiol-17β in the three different standards must be divided by four.

The standard mixtures were evaporated under nitrogen at 60 °C and the residues were dried under vacuum in a desiccator. The heptafluorobutyric ester derivatives were prepared as described above.

It should be noted that the exact weight of [4-$^{14}$C]oestradiol-17β in standards and samples is not important. However, it is necessary that the amount of [4-$^{14}$C]oestradiol-17β in the standards is exactly the same or an exactly known multiple of that added to the serum samples. This was achieved by using the same syringe with the repeating adaptor for spiking the serum and preparing the standards. Moreover, the amount of non-labelled oestradiol-17β in the standard samples must be exactly known.

### Gas Chromatography-Mass Spectrometry

With the LKB instrument, 20 µg of oestriol-pentafluoropropionate was introduced into the ion source of the mass spectrometer, using a direct inlet system. The magnetic field was adjusted to the molecular ion of the oestriol derivative (m/z 726). This molecular ion is used to continuously correct, by means of the computer, the temperature-dependent drift of the magnetic field strength during the actual recordings of the oestradiol-17β specific m/z-values. The high voltages necessary for the recording of these m/z-values (664 and 666) are calculated by the computer and adjusted by means of a digital-analogue converter.

With the Finnigan instrument the spectrometer was respectively scanned during gas chromatography over two small mass ranges (663.750 to 664.250 and 665.750 to 666.250) which include the m/z-values to be recorded. It was thereby ascertained that the spectrometer records the peak maxima even if the mass calibration of the instrument is drifting during the day.

With both instruments, the data obtained during gas chromatography at the oestradiol-17β specific m/z-values are continuously recorded by the computers and stored on magnetic discs. The signal for each of the m/z-values is integrated over a period of 140 ms with both instruments. For the final inspection of the peak shapes, the selected ion monitoring chromatograms are reconstructed by the computers and made visible on a display. Quantitative evaluation of peak heights was carried out either manually with a cross hair cursor or automatically by the computer programmes.

The mixture containing the oestradiol-17β derivative was injected into the gas liquid chromatography column, which was connected to one of the two mass spectrometry systems. Since the amount of substance available for the analysis of oestradiol-17β is rather low (pg-range), the complete sample must be injected into the gas chromatograph-mass spectrometer to obtain an accurate recording. This was accomplished by dissolving the residue of the derivative formation, which was carried out in small tapered test tubes, in approximately ten microliters of cyclohexane. The solution was aspirated into a 10-microliter-syringe and transferred in quantities of 2–3 microliters to the tip of the glass needle of the solid injection system (5). The solvent of each portion was evaporated to dryness within the moving-needle-solid-inlet system. After the total sample had been concentrated on the tip of the needle, which takes about 1–2 min, the substance was injected by rapidly moving the glass needle into the hot space (300 °C) just ahead of the column.

In order to keep the measurement system under continuous control it is of utmost importance that the serum and standard samples are injected alternately.

A selected ion recording as printed by the computer after processing a serum sample is shown in figure 3.

### Calculation Procedure

The concentration of oestradiol-17β in the serum samples was calculated according to a procedure, the principles and details of which have been outlined in the first paper of this series on the measurement of cortisol (4). The BASIC computer programme depicted therein is also applicable to the calculation of the concentration of oestradiol-17β.

## Accuracy

The accuracy of the method reported herein is based on the application of the highly specific method of selected ion monitoring combined with the isotope dilution principle, which provides the most suitable means for control of recovery.

## Precision

The precision of the method was calculated as Coefficient of Variation (CV) from a series of measurements of oestradiol-17β in a serum sample on different occasions. Sixteen different serum pools were investigated and the results have been summarised in table 1.

The overall imprecision was in the range 1.0 to 2.5%. This random error is assumed to be derived from several sources of imprecision:

(1) Isotope ratio measurement (0.5–1.0%, C.V.),
(2) Pipetting of serum samples (~ 0.1%, C.V.),
(3) Pipetting of the labelled internal standard solution (~ 0.4%, C.V.).

Tab. 1. Parameters of precision of the isotope dilution-mass spectrometry method for the determination of oestradiol-17β in human serum.

| Serum pool | Oestradiol-17β (nmol/l) | n | Standard Deviation (nmol/l) | Coefficient of Variation (%) |
|---|---|---|---|---|
| P. A. 77 | 0.319 | 8 | 0.0058 | 1.8 |
| P. B. 77 | 1.10 | 8 | 0.0280 | 1.9 |
| Be 2241 | 0.382 | 8 | 0.0059 | 1.5 |
| Be 2242 | 1.85 | 8 | 0.0230 | 1.3 |
| P. 7 | 1.015 | 6 | 0.0120 | 1.2 |
| P. 8 | 1.361 | 14 | 0.0297 | 2.2 |
| P. 9 | 0.964 | 8 | 0.0152 | 1.6 |
| P. 10 | 0.836 | 8 | 0.0087 | 1.0 |
| P. 11 | 1.133 | 8 | 0.0190 | 1.7 |
| P. 12 | 0.794 | 13 | 0.0120 | 1.5 |
| P. 13 | 1.169 | 16 | 0.0289 | 2.5 |
| S. 14 | 2.06 | 15 | 0.0476 | 2.3 |
| S. 15 | 3.53 | 10 | 0.0690 | 2.0 |
| S. 16 | 2.78 | 9 | 0.0400 | 1.4 |
| S. 17 | 1.364 | 10 | 0.0230 | 1.7 |
| S. 18 | 0.731 | 10 | 0.0120 | 1.6 |

## Sensitivity

The mass spectrometers used in this investigation were capable of measuring 5 pg oestradiol-17β with a signal to noise ratio of 3:1.

## Discussion

The sensitivity and specificity of selected ion monitoring are improved by selecting a derivative, the mass spectrum of which exhibits an ion of high relative intensity at a high m/z-value. As may be seen from the mass spectrum in figure 2, the heptafluorobutyric ester of oestradiol-17β proved to be the most suitable derivative with a molecular ion at m/z 664. By introducing two heptafluorobutyric groups the molecular weight of the phenolic steroid is increased from 272 to 664. At this high m/z-value there is only little interference, if any, to be expected from accompanying biological material and from the continuous bleeding of the gas liquid chromatography column, as may be seen from the selected ion recording in figure 3. The specificity of the method is further improved by the use of fused silica capillary columns in gas liquid chromatography.

The high sensitivity of the method using the heptafluorobutyric ester derivative may be explained from the fairly high abundance of the molecular ion at m/z 664, which forms the base peak of the mass spectrum. The lower limit of detection of the mass spectrometers used here is approximately 5 pg oestradiol-17β. Therefore, the procedure may be used for the measurement of oestradiol-17β in biological materials ranging from 20–30 pg with a fairly high degree of reliability. However, for the definitive measurement, the use of sufficient serum to provide at least 200 pg in each individual sample is recommended.

In view of the low concentration of oestradiol-17β in normal human serum, the total amount of the oestradiol-17β derivative originating from one sample should be subjected to combined gas chromatography-mass spectrometry. This is achieved by using the splitless moving needle solid injection technique (5).

Phenolic steroids in general are sensitive to irreversible adsorption by glassware during the analytical procedure; for this reason α-naphthol was added as a carrier substance to the initial sample as well as to the eluate of the Sephadex column.

For the development of a definitive method, the pure analyte as well as a suitable isotopically labelled compound of the same chemical structure are necessary prerequisites. As primary reference material, an

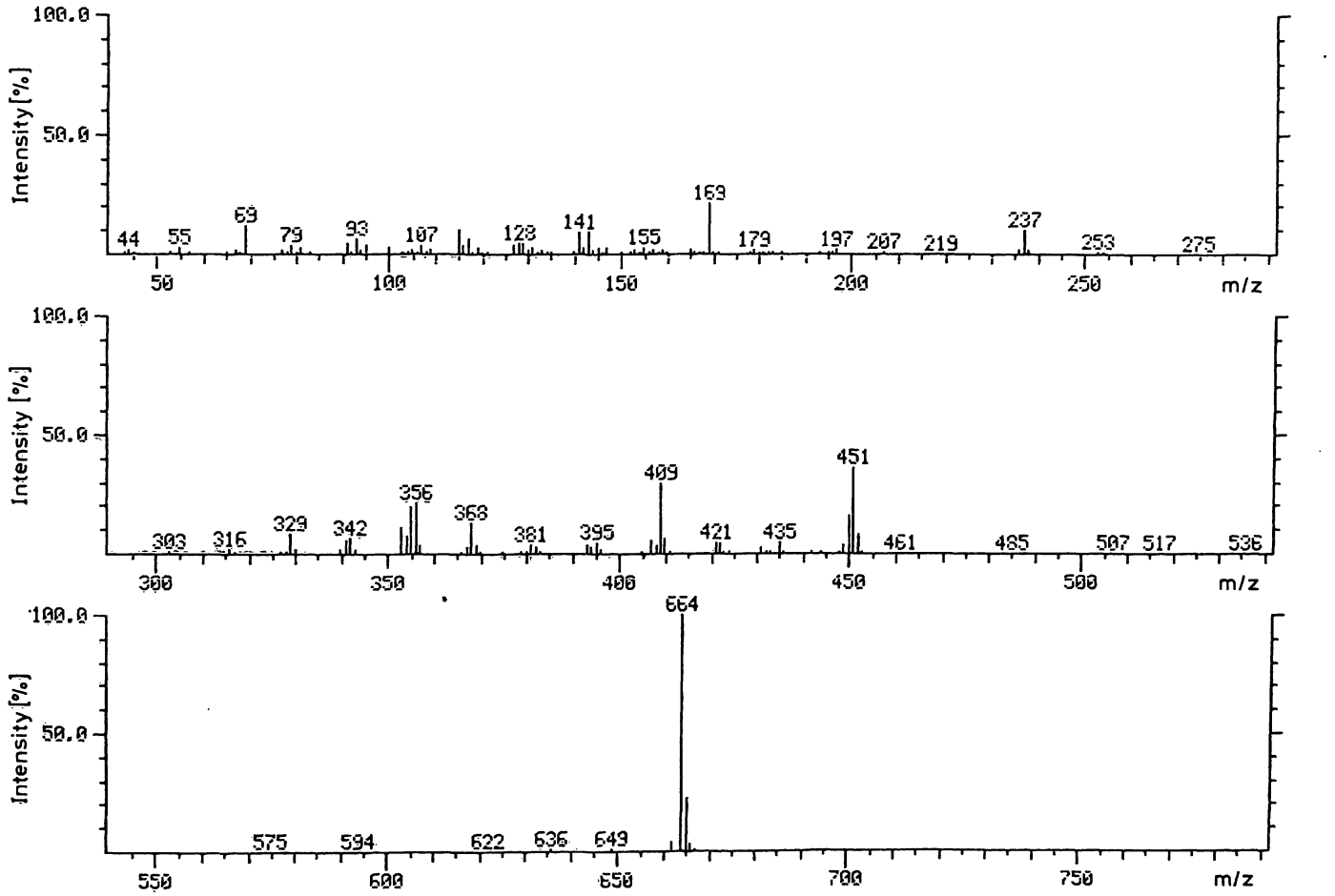


Fig. 2. Mass spectrum (computer print) of the heptafluorobutyric ester of oestradiol-17β. Mass spectrometer: LKB 9000; direct inlet probe: 45 °C; ion source: 20 eV, 60 μA, 250 °C.

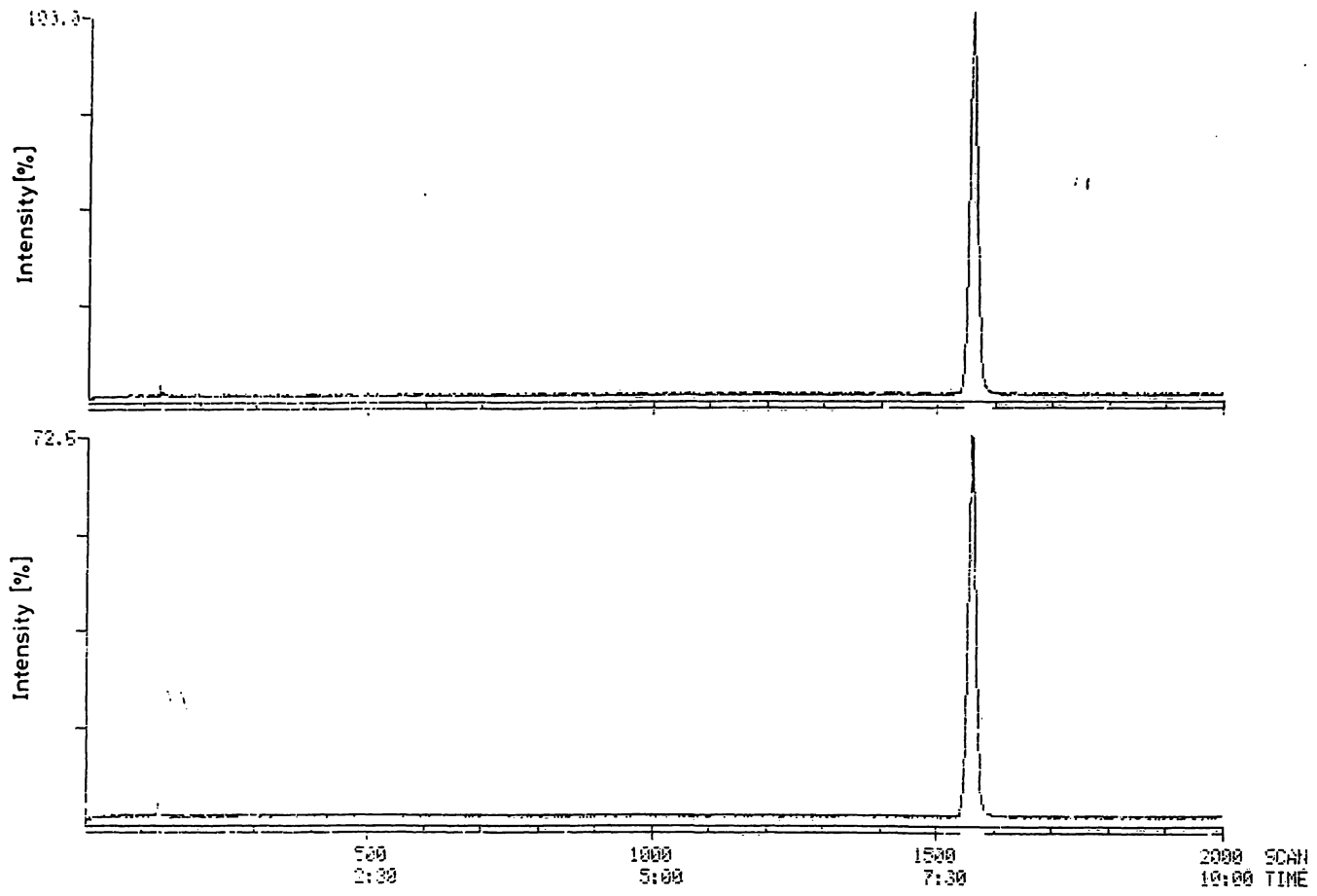


Fig. 3. Selected ion recording (computer print) of the heptafluorobutyric ester derivative of oestradiol-17β (lower trace) and [4-$^{14}$C]oestradiol-17β (upper trace) after processing a serum sample.

oestradiol-17β preparation was used which was prepared for a project of the Community Bureau of Reference of the European Communities.

$^{14}$C-Labelled oestradiol-17β was selected in the present study as internal standard material in order to avoid any risk of the label being changed during the analytical procedure, as may happen with the tritium or deuterium labelled compounds recommended by several investigators (6–10). The $^{14}$C-labelled steroid can be replaced in the future by the non-radioactive $^{13}$C-labelled oestradiol-17β which at present is not yet commercially available. The steroid should contain a minimum of two or preferably more $^{13}$C-atoms in order to minimise interference with the naturally occuring isotopes during selected ion monitoring.

In order to improve the precision of the method, the final measurement of the definitive values should be carried out at an isotope ratio of about 1. This ratio is obtained by adjusting the volume of the plasma sample rather than the amount of internal standard [4-$^{14}$C]oestradiol-17β.

The hierarchy concept of methods in clinical chemistry as outlined in the "Approved Recommendation (1978) on Quality Control in Clinical Chemistry" (3), defines a definitive method as a procedure which, after exhaustive investigation, is found to have no known source of inaccuracy or ambiguity. In practice, there is no final proof of accuracy even for a definitive method. It should be mentioned that some possible sources of error still remain. For example, the primary reference oestradiol-17β may contain up to 0.5% of impurities.

A further source of error may lie in the inaccuracy of the test weights for calibrating the balances, which is probably less than 0.5%. Finally a random error of 1.0 to 2.5%, arising from the imprecision of the isotope ratio measurement and the pipetting of serum and standards is associated with each individual analytical result. Nevertheless, it may be stated that due to the high specificity of the selected ion monitoring technique and the accurate control of recovery by the isotope dilution principle, the results obtained by the present method represent the nearest approximation to the true value.

Following the preliminary report of this technique (11) the method has now been in use in the laboratory for more than five years. Oestradiol-17β has been determined regularly in serum and plasma pools used for quality control surveys of the Deutsche Gesellschaft für Klinische Chemie. The definitive values for oestradiol-17β as well as for five further steroid hormones, have provided the basis for the evaluation of the results of the participants in the collaborative surveys for hormones (1). This is in accordance with a concept that has recently been proposed for the evaluation of collaborative surveys in clinical chemistry by using definitive or reference method values (12).

## Acknowledgements

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