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GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF ENDOGENOUS LEVELS OF ESTRADIOL IN PLASMA AND IN CYTOSOL FROM RAT UTERUS

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SUMMARY

A method that permits the analysis of low levels of estradiol in plasma from women, men and rats and in cytosolic fractions of uterine tissue is described. The samples are extracted with Amberlite XAD-2 and a phenolic fraction is isolated on a lipophilic ion exchanger. Less polar contaminants in this fraction are separated from estradiol on Sephadex LH-20. Estradiol from human plasma can then be analysed by gas chromatography–mass spectrometry (GC–MS) as the trimethylsilyl ether using a capillary column and single ion monitoring of m/z 416. Samples from rat plasma and uterine cytosol require final purification by high-performance liquid chromatography prior to the GC–MS analysis. The approximate detection limits with the GC–MS instrument used were $1 \cdot 10^{-11}$–$2 \cdot 10^{-11}$ moles/l in plasma and $5 \cdot 10^{-11}$ moles/kg in the uterus. Problems in the purification procedures and the specificity and sensitivity of GC–MS analyses are discussed.

INTRODUCTION

Studies of uptake, binding, nuclear translocation and further processing of steroid hormones in target organs require simultaneous analysis of endogenous levels of steroids and their receptors in subcellular compartments. As the hormonal response is elicited by very low concentrations of steroids, highly sensitive and specific methods are needed for the analysis. At present, gas chromatography–mass spectrometry (GC–MS) and immunoassays are the only methods that possess the necessary sensitivity. The specificity of immunoassays at picogram levels is very difficult to prove, and only indirect methods of validation have been used. With GC–MS, positive proof of specificity can be obtained.

In a previous paper[1] we described a simple and relatively rapid GC–MS method for the determination of estrogens in nuclear and cytosolic fractions from rat uterus. Estrogens were isolated and purified on a lipophilic ion exchanger, and an open-tubular glass capillary column was employed in the GC–MS analysis. The method was used to study levels of estradiol in blood and uterine nuclei after injection of estradiol. When it was applied to the analysis of endogenous levels of estradiol in blood and cytosol, the method of purification was found to be insufficient to ensure specificity for all types of samples.

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This study was carried out in order to achieve consistent purity of extracts for GC–MS analysis. Additional steps required for the analysis of estradiol in the phenolic fraction from plasma and cytosol are described. The methods are compatible with previously developed procedures[1,2] and can be applied to different types of biological samples.

EXPERIMENTAL

Glassware, solvents, reagents

All glassware was silanized with 5% dimethyldichlorosilane in toluene. Cleaning was carried out in an ultrasonic bath.

Solvents were of reagent grade and were re-distilled twice in an all-glass system with a 1-m distillation column. Spectroscopic-grade *n*-hexane (Merck, Darmstadt, G.F.R.) was used as supplied. Methanol was purified by addition of 0.4 volumes of 1.4 *M* sodium hydroxide solution in re-distilled water, refluxing for 6–8 h and distillation twice. Hexamethyldisilazane (Fluka, Buchs, Switzerland) and trimethylchlorosilane (Applied Science Labs., State College, PA, U.S.A.) were distilled. Pyridine was refluxed for 3–4 h over calcium hydride and distilled.

To remove non-volatile interfering components formed upon mixing of the reagents, trimethylchlorosilane, hexamethyldisilazane and pyridine were mixed in the proportions 1:2:2 (v/v/v) and heated at 60°C for 30 min. The mixture was then distilled first at 60°C and then at 110°C, small portions of the distillate being discarded at the beginning and before the temperature rise. The distillates collected at the two temperatures were combined and used as the silylating reagent. When kept in a stoppered glass tube in the dark, the reagent was stable for more than 3 months.

Steroids

Unlabelled estradiol, estrone and cholestane were obtained from Steraloids (Wilton, NH, U.S.A.). Ethynylestradiol was a gift from Professor B. Högberg (Leo, Hälsingborg, Sweden). [2,3,6,7(*n*)-^{3}H]Estradiol (85 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.) and was purified on a small column of triethylaminohydroxypropyl-Sephadex LH-20 (TEAP-LH-20) and by high-performance liquid chromatography (HPLC) prior to use. Radioactivity was determined in an Minibeta Model 1211 liquid scintillation counter (LKB, Bromma, Sweden) using Instagel (Packard, Downers Grove, IL, U.S.A.) as the scintillation liquid.

Chromatographic materials

Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA, U.S.A.) was extensively washed with acid, base and solvents and stored in distilled ethanol until required for use[3]. Lipidex 1000 and 5000 (Packard) were washed with aqueous and absolute ethanol at 70°C with stirring and were kept in distilled methanol until required[4]. Octadecylsilane-bonded silica (Sep-Pak C_{18}) was purchased in cartridges from Waters Assoc. (Milford, MA, U.S.A.). To eliminate contaminating compounds, the packing was removed from the cartridge and used in a glass column.

Sulphohydroxypropyl Sephadex LH-20 (SP-LH-20) (ion-exchange capacity 1.46 mequiv./g) was synthesized according to ref. 5 and stored in the Na^+ form in distilled methanol at 4°C. Before use, it was converted into the H^+ form by washing with 0.2 *M* hydrochloric acid in 72% aqueous ethanol.

TEAP-LH-20 (ref. 4) was kindly supplied by Dr. B. Egestad. It was stored in the acetate form at −20°C, and was converted into the OH^- form immediately before use by washing with 0.2 *M* sodium hydroxide in 72% ethanol.

Sephadex LH-20 (Pharmacia, Uppsala, Sweden) was sieved, and the fractions of 100–140 or 140–170 mesh were used. The material was extensively washed with dichloromethane, chloroform–methanol (1:1), ethanol and methanol with stirring at room temperature, and the moist gel was stored at 4°C until needed.

Glass columns of 8 mm I.D., having a solvent reservoir at the top and a water-jacket, were used with Amberlite XAD-2, Lipidex 1000 and Sep-Pak C_{18}. Columns without a water-jacket were used with SP-LH-20 (8 mm I.D.), SP-LH-20 and TEAP-LH-20 (4 mm I.D.). An end-piece of PTFE equipped with a stopcock was pushed into the lower end of the column. A piece of PTFE gauze (70 μm) on the end-piece supported the column bed[2].

High-performance liquid chromatography

HPLC was performed on an LDC instrument (LDC, Riviera Beach, FL, U.S.A.) using a column of LiChrosorb Diol (10 μm, 25 × 0.4 cm I.D.; Merck, Darmstadt, G.F.R.). A Rheodyne injector (Model 7125, Cotati, CA, U.S.A.) with a 1-ml sample loop was used, and the sample was injected with a 500-μl 500 A-RLC syringe (SGE, North Melbourne, Australia).

Gas–liquid chromatography (GLC)

GLC was performed on a Pye 104 gas chromatograph equipped with a flame-ionization detector and housing a 25 m × 0.2 mm I.D. open-tubular glass capillary column coated with SE-30 (ChromaChemie, Västerhaninge, Sweden). An all-glass solid injection system was used[6]. Nitrogen was used as the carrier gas at a pressure of about 50 kPa and the oven temperature was 260°C. Steroids were analysed as trimethylsilyl (TMS) ethers prepared by adding 60 μl of the distilled reagent mixture and heating at 60°C for 30 min. The sample was dried under nitrogen and dissolved in *n*-hexane.

Gas chromatography–mass spectrometry (GC–MS)

A modified LKB 9000 instrument was used with an open-tubular glass capillary column (25 m, SE-30; GC[2] Ltd., Northwich, Cheshire, Great Britain) connected to the ion source via a single-stage adjustable jet separator[2]. The column temperature was about 250°C and the temperatures of molecular separator and ion source were 250 and 290°C, respectively. The energy of the bombarding electrons was 22.5 eV, the ionizing current 120 μA and the accelerating voltage 3.5 kV.

Estradiol was determined by single-ion monitoring using the molecular ion of the derivative at *m/z* 416. Estradiol di-TMS ether, usually 8–16 pg, was used as the external standard and was injected 1–2 min before and after the injection of the sample. In this way the non-specific carrier effect of the biological sample was partly compensated for[1,7]. Cholestane (40–80 ng per injection) was added to all samples and standards, and the height of its peak in the total ion current chromatogram served to correct for differences in the aliquots injected.

Analytical procedures

In all analyses, [^{3}H]estradiol ($10 \cdot 10^3$–$25 \cdot 10^3$ dpm) was added to the samples,

and radioactivity was determined on aliquots of fractions collected during the purification procedure.

Estradiol in rat uterine nuclei. This was analysed as previously described[1].

Estradiol in human and rat plasma. Similar principles were used as in the method described previously[1]. Two adsorbents, Sep-Pak C_{18} and Amberlite XAD-2, were compared for the extraction. Plasma (2–4 ml) was diluted with one volume of physiological saline and sonicated for 5 min. The sample was applied to the columns of Sep-Pak C_{18} (10 × 8 mm) or Amberlite XAD-2 (50 × 8 mm) heated at 64°C (ref. 2) for 5 min by closing the stopcock, and was then percolated through Sep-Pak at a flow rate of 0.5 ml/min, and through Amberlite XAD-2 at a rate of 0.2 ml/min. The columns were rinsed with 10 ml of water at 64°C. The steroids were eluted with 6 ml (Sep-Pak) or 10 ml (Amberlite XAD-2) of methanol at room temperature at a flow rate of 0.5 ml/min. The Amberlite column was washed with 3 ml of *n*-hexane prior to this elution. The methanol eluate was directly passed through SP-LH-20 and TEAP-LH-20 as described previously[1]. The phenolic estrogen fraction from TEAP-LH-20[1] was taken to dryness under a stream of nitrogen. The residue was carefully dissolved in 400 μl of chloroform and 600 μl of *n*-hexane were then added. A 1-ml volume of sample was applied to a column of Sephadex LH-20 (20 × 8 mm, 0.45 g) at a flow-rate of 0.08 ml/min. The column was rinsed with 19 ml of *n*-hexane–chloroform (6:4) at a flow-rate of 0.15 ml/min. Estradiol was then eluted with *n*-hexane–chloroform–methanol (6:4:1) at the same flow-rate. The first 2 ml of the effluent were discarded and the subsequent 6–8 ml were collected and taken to dryness under a stream of nitrogen. In most analyses of human plasma the sample could then be derivatized and analysed by GC–MS.

In analyses of rat plasma, the sample had to be further purified by HPLC. The residue was dissolved in 40 μl of isopropanol by sonication, and 160 μl of *n*-hexane were added and mixed with a vortex mixer. The solution was aspirated into the 500-μl syringe and the tube was rinsed with 100 μl of 20% isopropanol in *n*-hexane, which was also aspirated into the syringe. The sample was injected and elution performed with *n*-hexane–dichloromethane–isopropanol (95:5:10) at a flow-rate of 1.2 ml/min. The volume between 11.4 and 15.3 ml of effluent was collected in a glass-stoppered centrifuge tube. Solvents were removed and the sample was derivatized and analysed by GC–MS.

Between sample applications, the column was washed with 20% methanol in dichloromethane for 10 min at a flow-rate of 3 ml/min, and was then conditioned with the mobile phase for 10 min at the same flow-rate.

Each day and when new batches of solvent were prepared, the retention time and reproducibility were checked by injection of trace amounts of [^{3}H]estradiol. Injection of estradiol for detection by UV absorption should be avoided because of the risk of memory effects in the picogram range.

Estradiol in rat uterine cytosol. A cytoplasmic fraction of uterine tissue was prepared as described previously[1] using a smaller volume of buffer. The steroids in 2–3 ml of the 0.01 *M* Tris–hydrochloric acid buffer (pH 7.9), with EDTA, were extracted with Amberlite XAD-2 or Lipidex 1000. The sample was diluted with one volume of saline and the pH was adjusted to 4 with 0.2 *M* hydrochloric acid. The extraction procedure using Amberlite XAD-2 was the same as for plasma. Lipidex 1000 (1–2 g, 70 × 8 mm in methanol) was washed with 20 ml of water before use. The diluted sample was applied to the gel bed, heated at 64°C for 5 min and passed

through the bed at a flow-rate of about 0.3 ml/min at 64°C. The bed was washed with 10 ml of water at the same flow-rate and temperature, and steroids were eluted with 8 ml of methanol at a flow-rate of 0.4–0.5 ml/min at room temperature. A phenolic fraction was isolated, and estradiol was purified by chromatography on Sephadex LH-20, and by HPLC, as described for plasma.

RESULTS AND DISCUSSION

Estradiol in uterine nuclei

The reproducibility of the method for the purification of estrogens in the nuclear fraction was assessed previously[1]. In the present study, the final recoveries of [^{3}H]estradiol added to the nulcear fractions of human endometrium and rat uterus were 87.3 ± 4.4% (mean ± S.D.; n = 23) and 89.4 ± 2.8% (n = 38), respectively, which is consistent with the previous report[1]. The purity of the samples was sufficient for the analysis of estradiol without interference by other compounds. As previously reported[1], several hundred picograms were present in the nuclear fraction from one rat uterus (Table I). These high concentrations and the relative purity of the isolated nuclei decrease the need for additional chromatographic steps to ensure specificity in the GC–MS analyses.

Estradiol in plasma and cytosol

Extraction and isolation of a phenolic fraction. In comparison with Amberlite XAD-2, Sep-Pak C_{18} has a higher capacity and extraction takes place more rapidly[8,9]. A comparison was made between the two adsorbents, particularly with respect to their influence on subsequent purification steps.

In the first experiments, pooled plasma from men was used. Labelled estradiol was extracted with Sep-Pak C_{18} at different pH values (5.5–10.5). Recoveries were 90.5 ± 3.6% (n = 6) and were not influenced by pH. Losses in the sample effluent, the water wash and on the column were 3.6 ± 0.5, 2.1 ± 0.3 and 2.3 ± 0.1%, respectively. The extracts were purified on SP-LH-20 and TEAP-LH-20 and recoveries in the

TABLE I

AMOUNTS OF ESTRADIOL IN THE NUCLEAR AND CYTOSOLIC FRACTIONS OF UTERI FROM 8 RATS IN DIFFERENT STAGES OF THE ESTRUS CYCLE

Uterine weights varied between 250 and 400 mg.

Rat No.	*Estradiol per uterus (pg)*		*Distribution ratio, nuclei/cytosol*
	Nuclei	*Cytosol*	
1	432	136	3.2
2	434	204	2.1
3	514	190	2.7
4	245	32	7.7
5	340	56	6.1
6	559	129	4.3
7	241	60	4.0
8	400	18	22.2

phenolic fraction from the latter column were 78.0 ± 7.0% (n = 6). However, this fraction was not sufficiently pure for analysis of picogram amounts of estradiol by GC–MS.

After testing the additional purification procedures as described below, attempts were made to quantitate estradiol in plasma from 19 women in different stages of the menstrual cycle. Purification steps consisted of Sep-Pak C_{18} extraction, filtration through the ion exchangers and SP-LH-20. Recoveries of ^{3}H-labelled steroids carried through the entire procedure were low and variable [45.1 ± 12.6% (n = 19)], as were recoveries in the phenolic fraction from TEAP-LH-20 [53.2 ± 5.3% (n = 4)].

These results showed that there were individual differences in recoveries, depending on the source of the sample. To investigate whether losses occurred in the Sep-Pak C_{18} extraction or in subsequent purification steps, five of the previously analysed samples from women were randomly selected for further studies. Recoveries of estradiol in the Sep-Pak C_{18} extraction were 90.0 ± 1.8% (n = 5), whereas recoveries in the phenolic fraction from TEAP-LH-20 were again low and variable [53.4 ± 24.6% (n = 4)]. The missing radioactivity was not found in any of the fractions analysed, and SP-LH-20 or glass surfaces remained the only possible sites of major loss. There appeared to be a loss associated with storage of fractions in methanol.

At this stage of the study, all glassware was not silanized, as the experiments with the pool of plasma from men indicated that this was not necessary. Following the poor results with plasma from women, all glassware was silanized, and a comparison was made between the use of this and untreated glass. Samples were studied that had previously given poor recoveries of estradiol, and both Amberlite XAD-2 and Sep-Pak C_{18} were tested. Large losses occurred with these samples when the glass was not silanized. Thus, using Sep-Pak C_{18} for extraction, recoveries were 31% and 46% for one and 36% and 65% for another sample run in duplicate through the procedure, including Sephadex LH-20. Losses increased with increasing time of storage in methanol (3–7 days). When silanized glass was used, recoveries were 73% and 69% for one sample and 71% and 66% for the other, and losses were not related to storage time (0–3 days). When Amberlite XAD-2 was used instead of Sep-Pak C_{18}, recoveries from the same samples carried through the same procedure were higher, 80% and 83%. The influence of the extraction procedure on the recoveries in subsequent purification steps cannot yet be explained, but based on these results Amberlite XAD-2 is preferred for the extraction of estradiol in plasma.

Studies of the extraction of cytosolic fractions were performed essentially as with plasma. Lipidex 1000 was tested as an alternative adsorbent[10]. The influence of the extraction method on losses in subsequent purification steps was observed as with plasma. Thus, preliminary studies using non-silanized glassware and purification including Sephadex LH-20, showed recoveries of 66, 52 and 37% in the estradiol fraction when Lipidex 1000, Amberlite XAD-2 and Sep-Pak C_{18}, respectively, were used for extraction. Losses in the aqueous phase from Amberlite XAD-2 were large (10.9 and 14.6%), possibly influenced by the high pH of 7.9. When the extractions were carried out at pH 4, losses in the aqueous phases from the columns of Lipidex 1000 and Amberlite XAD-2 were 6.0 ± 5.3% (n = 4) and 3.4 ± 1.1% (n = 4), respectively. One of the samples extracted with Lipidex was responsible for the larger loss with this method.

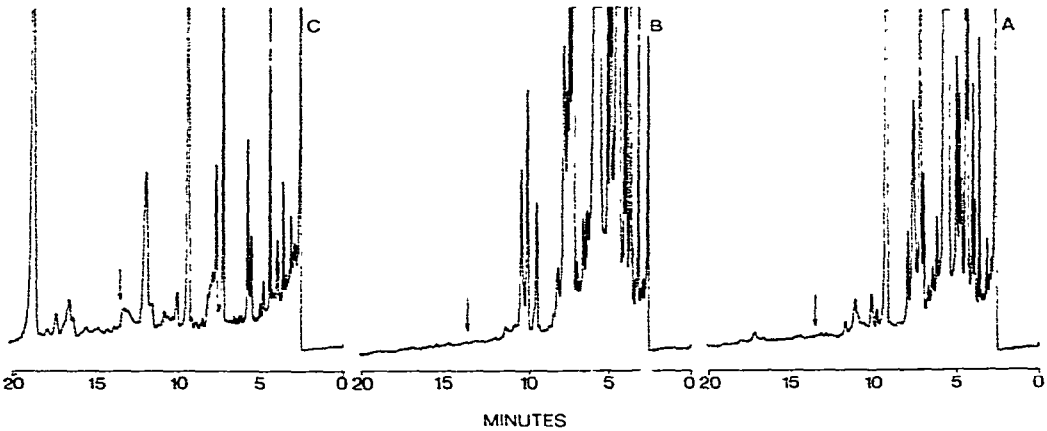

Fig. 1. GLC analyses of fractions collected from the column of Sephadex LH-20 in the purification of 4 ml of pooled plasma from men. A, 0–4 ml; B, 4–8 ml; C, 22.5–27.5 ml (estradiol fraction). Aliquots equivalent to 80 μl of plasma were injected. Cholestane was added to the estradiol fraction (peak of 20 ng at 18.5 min). The retention time of estradiol TMS ether is indicated by an arrow.

Chromatography on Sephadex LH-20. As additional purification of the phenolic fraction was needed for the analysis of low endogenous levels of estradiol, chromatography on Sephadex LH-20 was tested[11,12]. The solvent system and column dimension were optimized in terms of solubility of the sample and minimum loss of radioactivity in the fraction eluted before estradiol. *n*-Hexane–chloroform (6:4) was used for sample application and elution of less polar compounds, and *n*-hexane–chloroform–methanol (6:4:1) for elution of estradiol. Column dimensions of 20 × 8 mm I.D. were sufficient to avoid significant leakage of steroid with the void volume. As the gel swelled more in the second solvent, the column had to be wide and short in order to maintain a suitable flow-rate. To determine the minimum volume for elution with the first solvent, extracts of plasma from rats and humans were applied, and 4-ml fractions were collected. The purity of each fraction was examined by GLC (Fig. 1). Elution with 20 ml of solvent was found to be needed for the removal of less polar impurities. The recovery of estradiol was more than 96% in 4–5 ml of the second solvent. The losses in fractions collected before and after the estradiol fraction were 4.2 ± 2.3% (n = 9) and 1.7 ± 0.5% (n = 13), respectively, in analyses of plasma from women. Analyses of cytosol showed a loss of 1.5 ± 0.5% (n = 13) in the early fraction.

The purity of the estradiol fraction usually permitted solid injection of the equivalent of more than 0.4 ml of plasma (Fig. 2), which was sufficient for studies of basal levels of estradiol in plasma from women (Fig. 3). However, samples from rat plasma and cytosolic fractions required further purification (Fig. 4). Lipidex 5000 was tested in *n*-hexane–chloroform (8:2), but 10.8 ± 4.7% of [^{3}H]estradiol remained on the column following elution of the steroid with *n*-hexane–chloroform–methanol (8:2:0.5). Thus, Lipidex 5000 was inferior to Sephadex LH-20 in this purification step.

High-performance liquid chromatography. HPLC was tested as a final step in the purification procedure. Based on the studies of ethynylestradiol metabolites by Williams and Goldzieher[13], a straight-phase system using a LiChrosorb Diol column

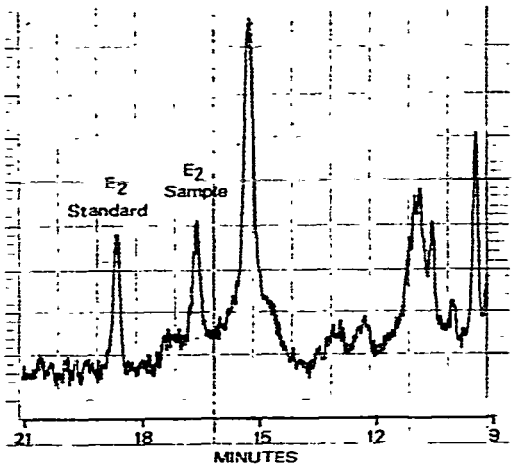

Fig. 2. GC–MS analysis of the estradiol fraction collected from Sephadex LH-20 in the analysis of 4 ml of pooled plasma from men (*cf.* Fig. 1). The equivalent of 0.6 ml of plasma was injected and *m/z* 416 was monitored. The peaks of estradiol TMS ether from the sample and the standard (8 pg) injected 2 min later are indicated. The temperature of the capillary column was 215°C to permit separation of the steroid from contaminating compounds with shorter retention times.

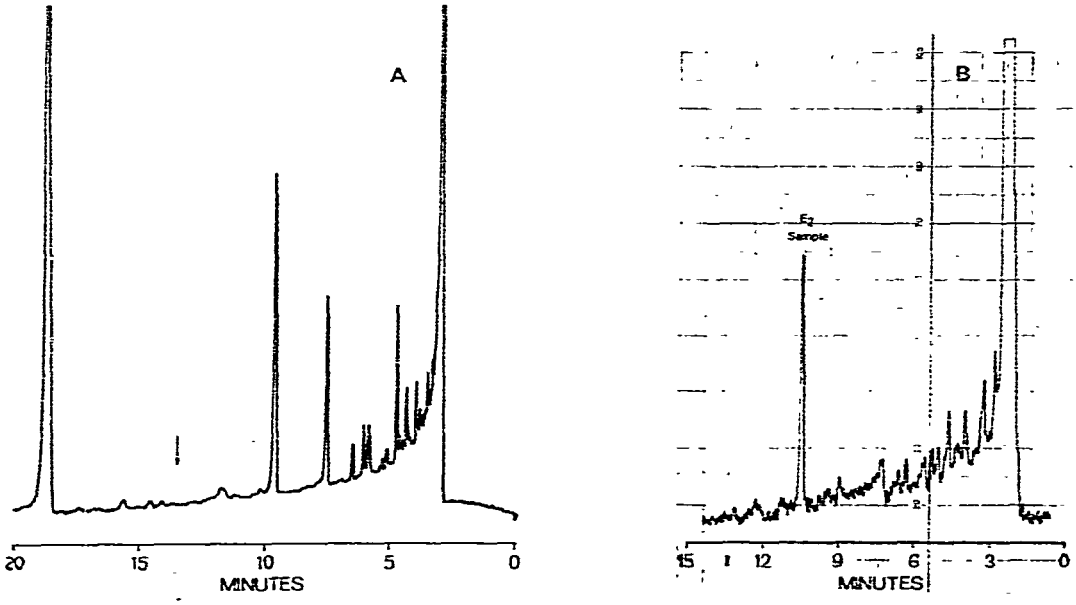

Fig. 3. (A) GLC and (B) GC–MS analyses showing purity of the estradiol fraction from Sephadex LH-20 in the analysis of plasma from a woman. The equivalents of 40 and 160 μl of plasma were injected in A and B, respectively. Cholestane was added and the peak at 19 min in A represents 20 ng. The peak of estradiol TMS ether in B represents about 14 pg.

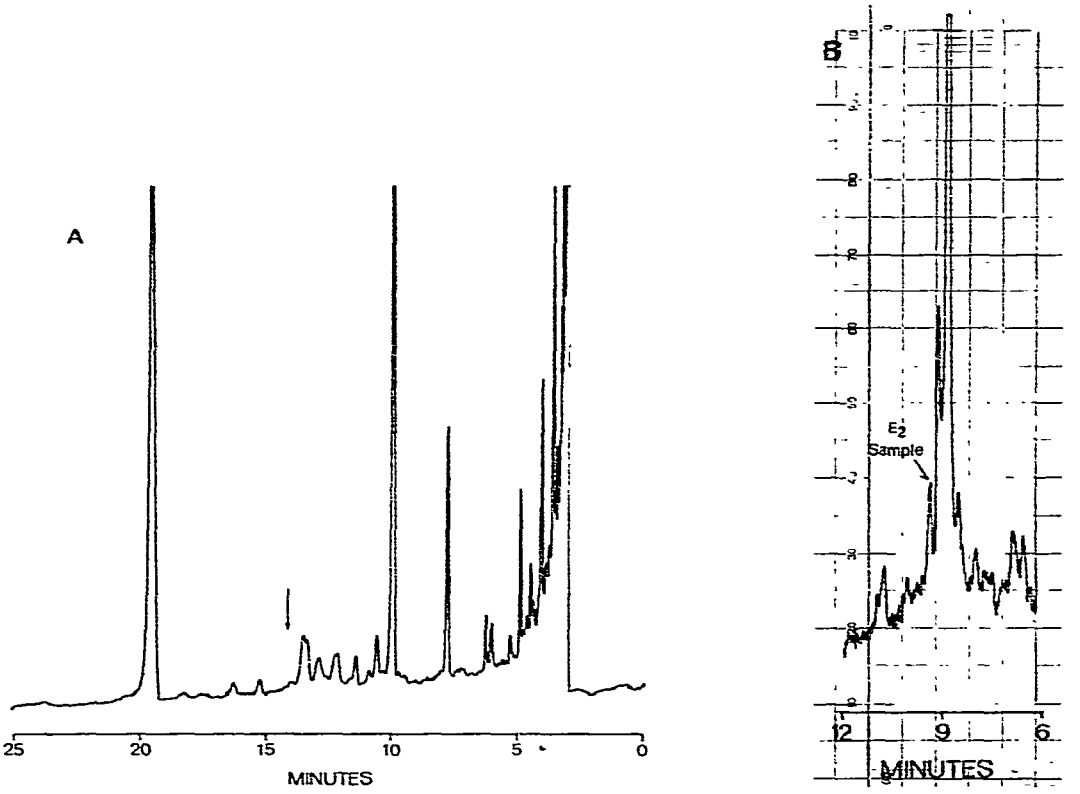

Fig. 4. (A) GLC and (B) GC–MS analyses showing insufficient purity of the estradiol fraction from Sephadex LH-20 in the analysis of a cytosol fraction from rat uterus. The equivalents of 2 and 8% of the uterus were injected in A and B, respectively. Cholestane was added and the peak at about 20 min in A represents 20 ng. The peak at the retention time of estradiol TMS ether in B might represent 6 pg of the compound.

was chosen. Solvent systems were tested for the separation of estrone, estradiol and ethynylestradiol, using UV detection. The solvent mixture *n*-hexane–dichloromethane–isopropanol (95:5:10), was optimal, with an uncorrected retention volume of estradiol of about 14 ml. Removal of polar contaminants remaining on the column required elution with 30 ml of 20% methanol in dichloromethane for 10 min. Another 10 min were required for equilibration with the mobile phase. Thus, samples could be injected at intervals of 40 min.

The volume and polarity of the solvent used for injection were important in obtaining consistent elution volumes and resolution factors. A maximum of 300 μl of 40% isopropanol in *n*-hexane could be used. Experiments with [^{3}H]estradiol showed that the steroid was quantitatively transferred to the column [1.9 ± 0.7% (n = 8) remained in the test tube]. There was no loss of estradiol on the column, 96% and 103% of the injected amount being recovered in two experiments. The degree of purity permitted solid injection of the equivalents of more than 0.2 ml of rat plasma and one third of a uterus without overloading of the capillary column.

The need for purification on Sephadex LH-20 prior to HPLC was tested. Three

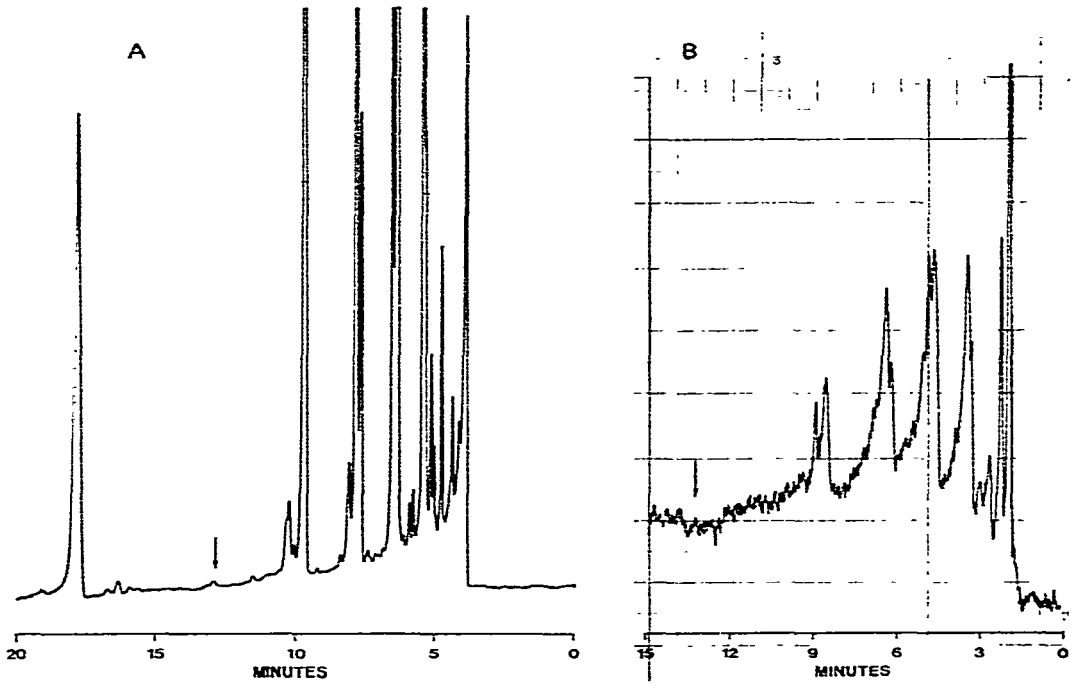

Fig. 5. (A) GLC and (B) GC–MS analyses of the effluent volume corresponding to estradiol, obtained in the HPLC separation of the non-polar contaminants from the Sephadex LH-20 column. The wide tailing peaks in B are presumably due to overloading with the four main components seen in A, resulting in increasing background from the stationary phase.

phenolic fractions from rat plasma and three from cytosol were separated on Sephadex LH-20 and the non-polar fraction (*i.e.*, before elution of estradiol) was collected. This material was subjected to HPLC and effluent corresponding to an estradiol fraction was collected. The purity of this fraction was evaluated by GLC and single-ion monitoring GC–MS. Peaks in the retention time range of estradiol TMS ether were not seen in the latter analyses but there was a marked rise of the baseline with all samples, particularly those from plasma (Fig. 5). A strong UV absorption of the effluent from the HPLC column also indicated that direct purification of the phenolic fraction from TEAP-LH-20 may lead to overloading of both the HPLC and capillary columns, with resulting rapid deterioration. Thus, purification on Sephadex LH-20 is recommended, especially when the steroid levels are close to the detection limit of the GC–MS instrument.

Evaluation of the complete method

Based on the studies described above, the original analytical procedure[1] was extended for the analysis of very low levels of estradiol. The method, including fractionation on lipophilic ion exchangers, Sephadex LH-20 and HPLC, was assessed by analysis of samples of rat plasma to which [^{3}H]estradiol was added. The final recoveries were 59.2 $\pm$ 8.9% in 12 experiments. The purity of the sample permitted the analysis of low endogenous levels of estradiol (Figs. 6 and 7). The detection limit was estimated to be 4–5 pg/ml, *i.e.*, about $1 \cdot 10^{-11}$–$2 \cdot 10^{-11}$ moles/l in plasma.

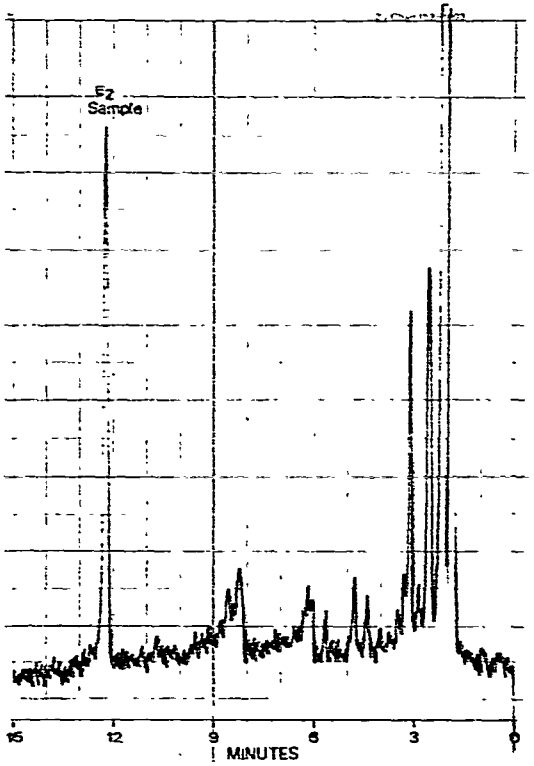

Fig. 6. GC–MS analysis of estradiol in a sample of female rat plasma showing purity of the final HPLC fraction. The equivalent of 0.12 ml of plasma was injected.

The method was tested in the same way for the analysis of estradiol in rat uterine cytosol. Final recoveries of radioactivity in the fraction from the HPLC column were 65.9 ± 13.8% (extraction with Lipidex; $n = 4$) and 74.3 ± 7.6% (extraction with Amberlite XAD-2; $n = 4$). The lower yield and higher standard deviation in the former extraction was caused by one sample.

The purity of samples carried through the entire procedure after extractions with Lipidex 1000 or Amberlite XAD-2 was evaluated by GLC and GC–MS with single-ion monitoring. The purity was sufficiently high (Figs. 8 and 9), and there was no difference between the two extraction procedures in this respect. As shown in Table I, the amount of estradiol in the cytosolic fraction from one rat uterus varied between 13 and 129 pg, which corresponds to concentrations of about $0.1 \cdot 10^{-9}$–$1 \cdot 10^{-9}$ moles/kg in tissue. The detection limit was estimated to be about 5 pg per uterus, *i.e.*, about $5 \cdot 10^{-11}$ moles/kg.

Specificity and sensitivity in steroid analysis

Specificity is the most important property of an analytical method. In this instance it is based on the selective isolation procedure and the use of a capillary column in the GC–MS analysis. Although only molecular ions were monitored, the absence of peaks in the vicinity of the estradiol derivative makes it unlikely that the

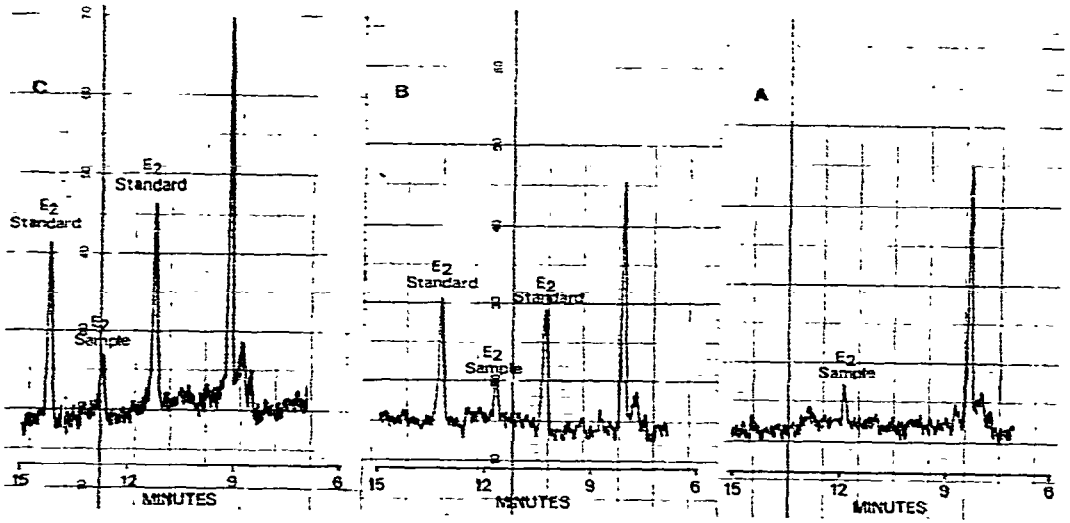

Fig. 7. GC–MS analyses of estradiol in two samples (A + B, and C), of plasma from female rats. The equivalents of 0.12 ml of plasma were injected. A without and B and C with injection of standards (8 pg) 1.5 min before and after injection of the samples.

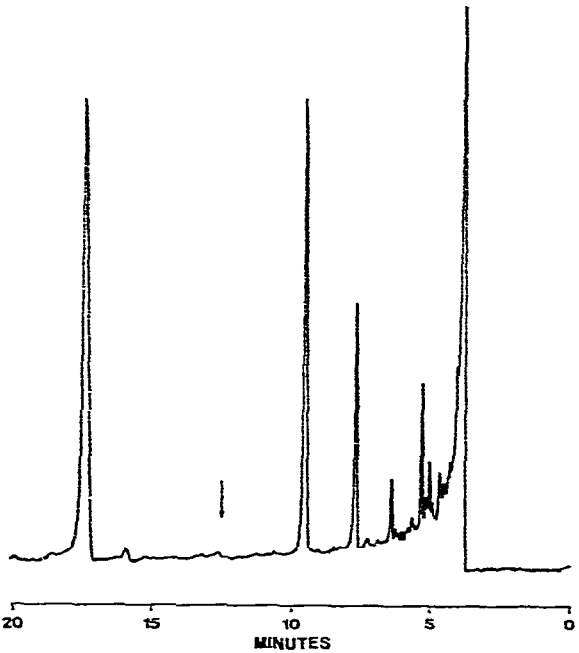

Fig. 8. GLC analysis showing the purity of the final HPLC fraction obtained in the analysis of estradiol in a sample of rat uterine cytosol. The equivalent of 8% of the uterus was injected. Added cholestane (16 ng) is seen at 17.5 min. The peak at 9.5 min is bis-(2-ethylhexyl) phthalate. The retention time of estradiol TMS ether is indicated by an arrow.

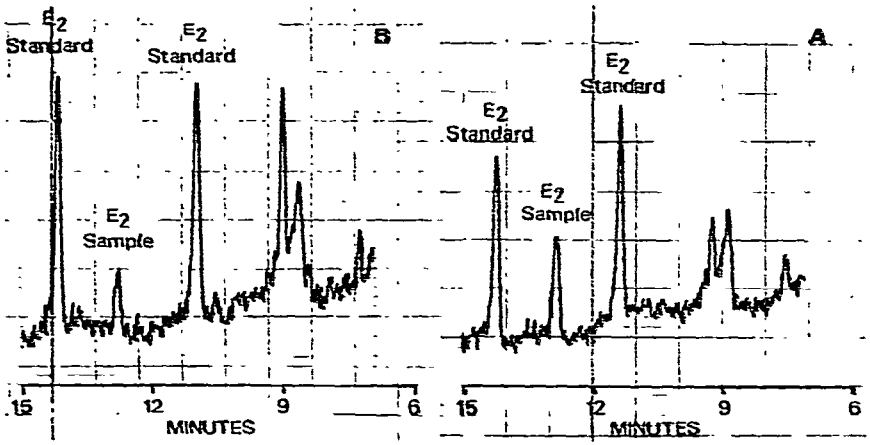

Fig. 9. GC–MS analyses of estradiol in two samples of rat uterine cytosol. The equivalents of 8% (A, same as in Fig. 8) and 12% (B) of the uterus were injected. The standards injected before and after the samples represent 16 pg of estradiol TMS ether.

peak with the appropriate retention time is due to another compound. The use of multiple ion monitoring would increase specificity at the expense of sensitivity. This method has been employed after addition of internal standards labelled with heavy isotopes in analyses of estradiol and other estrogens in plasma[14–19]. Our results indicate that packed columns used in most previous studies may not ensure sufficient specificity in many instances. However, the results of several studies indicate that loss of chromatographic resolution can be compensated for by use of derivatives that give more specific ions at high mass, *e.g.*, heptafluorobutyrates[14,16,18,19]. Another method is to use a high-resolution mass spectrometer as a detector[20–23]. Inherent in all GC–MS methods is the possibility of evaluating the specificity in each individual analysis. Immunoassays do not provide this possibility without additional analyses and considerable loss of sensitivity[24].

The purification has two objectives: to increase specificity and to permit injection of larger aliquots of sample in the capillary column. Contaminating compounds disturb the analyses even when they do not have the same retention time as the steroid being analysed. One effect seems to be on the background from the stationary phase. Thus, elution of 10–20 ng of bis-(2-ethylhexyl) phthalate results in the appearance of a peak with a sharp leading edge and a long tail. As the molecular weight of the compound is 390, this peak is probably due to increased elution of stationary phase which gives an ion of mass 415 with an isotope peak at *m/z* 416. The same displacement effect is noted with other contaminating compounds (*cf.*, Fig. 5). A general rise of the baseline is also seen with impure samples.

This study is part of a programme aimed at the development of generally applicable methods for the analysis of steroids[2–4] and metabolic studies using stable-isotope labelling[25]. Therefore, internal standards are not added, although this would result in considerably improved accuracy in analyses of individual steroids. This approach makes it important to achieve high recoveries in the purification procedure. The study illustrates the necessity for treating each individual sample as a separate specificity and sensitivity problem. The recovery of estradiol differed between sam-

ples. High recoveries were obtained with the sample of pooled plasma from men, irrespective of whether the glassware was silanized or not. When samples from women, rats and uterine cytosol were analysed, use of silanized glassware was essential for good recoveries in most instances. It is of interest that the losses were highest when extracts obtained with Sep-Pak C_{18} were carried through the purification procedure. Thus, the recovery is a complex function of the nature of the individual sample, the method used for extraction and the materials used in the handling of the extracts.

The combination of group separation by ion exchange[1,2] and isolation of individual steroids by partition chromatography results in highly purified samples. As more material can be analysed, sensitivity is increased. The purification procedure is relatively time consuming, 3 days being required including HPLC. Ten samples can be processed simultaneously. The advantages are that the capillary column is not overloaded and damaged by the sample injections and that a variety of steroids may be analysed with slight modifications of the final steps of the purification procedure.

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