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ION EXCHANGE PURIFICATION OF ESTROGENS

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SUMMARY

With the aim of developing a method for the determination of the whole estrogen profile in biological materials, including the labile estrogens, several alternative ion exchange chromatographic purifications have been assessed. A two-step anion exchange chromatography, first on a DEAE-Sephadex A-25 gel in the acetate form and then on the same gel in the hydroxyl form, gave the best results. The use of ascorbic acid and small columns (in Pasteur pipettes) combined with silanization of all glassware were essential for the preservation of the labile estrogens. The recovery of estrogen standards varied from 85 to 95% and no significant interconversions were detected. When estrogens were added to hydrolysed urine the recoveries were 50–90% and the extracts were pure enough for gas chromatography on capillary columns. These results can be improved by adding a further purification procedure prior to the ion exchange chromatography on DEAE-Sephadex A-25 in the hydroxyl form.

INTRODUCTION

Ion exchange chromatography has been employed for the purification and group separation of urinary estrogen conjugates [1–4] and the separation of free estrogens from neutral steroids [5–10]. Recently, Cohen *et al.*[11] utilizing the AG1-X2,HCO_3^- resin with ascorbic acid for protection of labile estrogens developed a method for the determination of about 12 estrogens in pregnancy urine with a detection limit of about 0.1 mg/day. The AG1-X2 resin has also been used in the purification of estrogens from feces for the determination of estrone, estradiol and estriol by radioimmunoassay in both women and men [12].

In the course of our attempts to develop a method for the determination of the whole estrogen profile in different biological materials we have tested a variety of ion exchange chromatographic materials. At the beginning of this study the AG1-X2 resin was used for this purpose. This resin gave good results for stable estrogens but when labile estrogens were chromatographed the results were not satisfactory. The term labile estrogens refers mainly to the catecholic and ring D-ketolic estrogens which are easily destroyed during the usual work-up procedures. Especially the catecholic 2- and 4-hydroxyestrogens which are important estrogen metabolites [13–16] undergo oxidative decompositon and are completely destroyed. To preserve them a number of other alternatives were tried and this report presents the results of these studies.

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EXPERIMENTAL

Steroids

The reference steroids were obtained from the following sources:

Ikapharm (Rhamat-Gan, Israel): estrone, 2-methoxyestrone, 2-methoxyestradiol-17β, estradiol-17β, 16α-hydroxyestrone, 16-oxoestradiol-17β, 6α-hydroxyestradiol-17β, 16,17-epiestriol, 17-epiestriol, estriol, 2-methoxyestriol. Steraloids (Wilton, N. H., U.S.A.): 16-epiestriol, 15α-hydroxyestriol. Imperial Cancer Research Fund (London, England): 2-hydroxyestradiol-17β. Mann Research laboratories (New York, N.Y., U.S.A.): 2-hydroxyestriol. 15α-Hydroxyestrone was a gift from Dr. Klaus Kieslich (Schering AG, Berlin, Germany) and 15α-hydroxyestradiol-17β, 2-hydroxyestrone and 4-hydroxyestrone were gifts from Prof. R. Knuppen.

Preparation of glassware

All glassware including Pasteur pipettes was silanized with 1% dimethyl dichlorosilane in toluene to prevent the possible creepage of estrogens and subsequent decomposition [17].

Recovery experiments

The experiments have been carried out with standards only or estrogens added to the extracts of 10 ml of gel-filtered and hydrolysed male urine, which was used because of the low estrogen background. The quantities of the added standards were in the range 1–3 μg (100–300 ng/ml urine). Thus the quantification was easily achieved by gas chromatography with open tubular glass capillary columns.

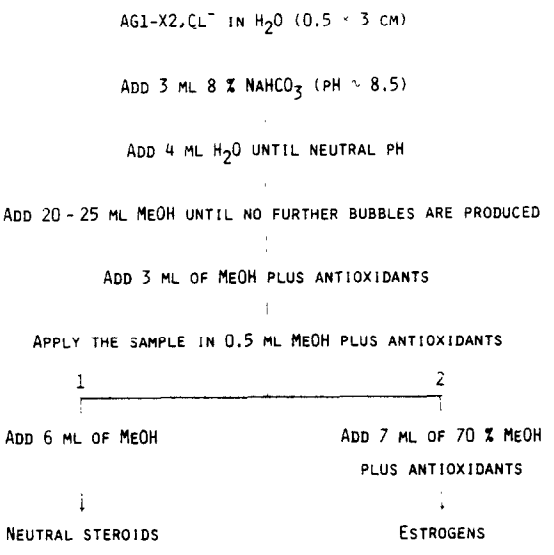

Fig. 1. Flow-diagram of the anion exchange chromatography on AG1-X2, HCO_3^-. Antioxidants: 0.01% ascorbic acid plus 1 mM dithiothreitol. Glassware was silanized.

Gel-filtration and hydrolysis

To economize on time, because of the large number of experiments, 300 ml of urine from normal men was gel-filtered on 4 × 50 cm Sephadex G-25 (medium) columns according to Beling[18]. Peaks I and II were hydrolysed separately with Helix pomatia extract (1000 FU/ml) for 16 h at 37°C after adding one ninth of the volume of 1.5 M acetate buffer, pH 4.1 [19]. The two peaks were hydrolysed separately because peak I contains more enzyme inhibitors, which results in lower yields [19]. Peaks I and II were combined immediately after hydrolysis and the total volume measured. This hydrolysate was then equally distributed into 30 tubes for extraction.

Extraction

The extraction was done using an equal volume of freshly distilled diethyl ether followed twice by half the volume. The washing of the extract was done once with 1/20 of the volume of 8% $NaHCO_3$ and twice with a similar volume of H_2O.

Ion exchange materials

AG1-X2 and Bio-Rex 5 resins were obtained from Bio-Rad laboratories (Richmond, California, U.S.A.) and DEAE-Sephadex A-25 from Pharmacia (Uppsala, Sweden). All these anion exchangers were available in the Cl^- form and had to be converted to the appropriate form prior to use.

AG1-X2 was washed with distilled water at room temperature and stored at +4°C. Conversion to the bicarbonate form was made immediately before the anion exchange chromatography (Fig. 1).

Bio-Rex 5 was also washed several times with distilled water at room temperature and stored at +4°C. Conversion to the acetate or free base form was done in a sintered glass funnel with vacuum suction immediately before use, in the following way. The resin was first washed with at least five resin bed volumes of 0.5 N acetic acid or 0.5 N NaOH in 72% ethanol and then with 20% ethanol until the pH of the eluate was 7. After washing with methanol the resin was suspended in this solvent.

DEAE-Sephadex A-25 was washed successively with 20%, 50% and absolute ethanol at 70°C. It was stored in 72% ethanol at +4°C. Conversion to the acetate and free base form was done immediately before use in the same way as described for the Bio-Rex 5 resin.

The conversion to the free base form of Bio-Rex 5 and DEAE-Sephadex A-25 anion exchangers is not complete and actually 20% of the available capacity has OH^- groups as counter anion (titrated by 0.1 N HCl). Therefore to indicate that the column has been washed with NaOH the term OH^- form will be used in the text.

Antioxidants

Ascorbic acid (Merck, Darmstadt, Germany) and dithiothreitol (DTT) (Calbiochem, San Diego, California, U.S.A.) were analytical grade. The solutions of antioxidants must be freshly made for optimal results. The concentrations used are mentioned in the figures. The removal of the antioxidants used was done just before the derivatisation of the steroids by adding 5 ml redistilled ethyl acetate to the evaporated sample and washing three times with 0.5 ml H_2O.

Derivatisation and purification of derivatives

Trimethylsilyl ether derivatives were made by adding 250 μl of a mixture prepared by mixing pyridine, hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMS) (0.9:0.3:0.1, by vol.) and incubating overnight or for 30 min in 60°C. Purification of the derivatives was done on a Lipidex-5000 column using *n*-hexane–HMDS–pyridine (98:1:1, by vol.) as eluant [20, 21].

Gas chromatography

Gas chromatography was carried out using a Carlo Erba instrument with an SE-30 open tubular glass capillary column and stigmasterol as internal standard.

RESULTS

Studies with the AG1-X2 resin

AG1-X2 (200–400 mesh) is a strongly basic anion exchange resin with a capacity 0.8 meq/ml of resin in H_2O. Experiments with the bicarbonate form showed that silanized glassware, small size of columns and ascorbic acid [22–26] are essential for the preservation of the labile estrogens (Table 1). Because Kaplan *et al.*[7, 8] has already used mini-columns 0.3 × 4.5 cm for 15 ml of urine we decided to use

Table 1. Summary of the recovery results obtained with the AG1-X2,HCO_3^- anion-exchange resin

	Standards only					Urine
Column size	1 × 10 cm	0.5 × 3 cm	0.5 × 3 cm	0.5 × 3 cm	0.5 × 3 cm	0.5 × 3 cm
Amount of estrogens added	15–20 μg	15–20 μg	15–20 μg	1–3 μg	1–3 μg	1–3 μg
Antioxidants	No	No	Yes	Yes	Yes	Yes
Silanized glassware	No	No	No	No	Yes	Yes
Stable estrogens	90–95%	90–95%	90–95%	90–95%	90–95%	~ 90%
Ring D-ketolic estrogens	10–15%	57%	80–90%	80–90%	80–90%	70–80%
2-Hydroxyestrogens	0%	0%	40–50%	5%	40–50%	30%

small columns 0.5 × 3 cm in short Pasteur pipettes. However, ascorbic acid could not be used in high concentrations because it effected the HCO_3^- of the resin decreasing the separation of the estrogens from the neutral steroids. Therefore a combination of 0.01% ascorbic acid with 1 mmol/l DTT was used. The procedure with AG1-X2 was as seen in Fig. 1 and the results obtained from this resin are shown in Table 1.

The purity of the gas chromatograms obtained with standards added to urine was rather good but extra peaks, sometimes quite pronounced were seen among the ring D-ketolic estrogen peaks. They probably represented organic acids (see later).

The same resin was tested in the OH^- form. This form permitted the use of higher concentrations of ascorbic acid but 70% methanol could not elute the estrogens out of the column. This was possible with 0.5% acetic acid in methanol but both the recoveries and the degree of purification were not satisfactory.

Studies with the Bio-Rex 5 resin

Bio-Rex 5 (200–400 mesh) is an intermediate base resin with a capacity of 2.8 meq/ml of resin bed in water. The OH^- form was able to sorb the estrogens but 70% methanol could not elute them. Therefore many different elution systems were tried and the best was found to be 0.5% acetic acid or 0.01 M LiCl in methanol. The results were the same as with the AG1-X2 resin but with the disadvantage of consistently low recoveries of 16α-hydroxyestrone and 16-oxoestradiol. Some nonspecific adsorption of these estrogens into the AG1-X2 resin occurs and has already been discussed [11], but obviously this property was more prominent with the Bio-Rex 5 resin. Another factor determining the degree of this nonspecific adsorption was the pH of the eluent. Elution with organic acids like acetic acid (acidic pH) gave better recoveries for 2-hydroxyestrogens than for 16α-hydroxyestrone and 16-oxoestradiol, while elution with 0.01 M LiCl gave the opposite result. LiCl was obviously converted to LiOH during the elution which gave a more basic pH destroying the 2-hydroxyestrogens. However, it seemed to diminish the nonspecific adsorption of 16α-hydroxyestrone and 16-oxoestradiol into the resin.

When urinary extracts were used the purity of the gas chromatograms was much better with LiCl than with acetic acid elution. With the latter high interfering peaks occurred in the area of the ring D-ketolic estrogens. These peaks, except for two small ones, disappeared when the urine extract was prepurified in a Bio-Rex 5,Ac^- column which is a weak anion exchanger (see later). Also this prepurification improved the recoveries obtained from Bio-Rex 5,OH^-.

Studies with DEAE-Sephadex A-25 gel

DEAE-Sephadex A-25 (200–400 mesh) is a weakly basic anion exchanger available in the Cl^- form, with a capacity of 3.5 ± 0.5 meq/g. It has already been employed for the separation of estrogen conjugates [1–3]. Though it is a weakly basic anion exchanger in the OH^- form it is able to retain the estrogens permitting their separation from the neutral steroids. It is also sufficiently stable when ascorbic acid is used. The chromatography on DEAE-Sephadex A-25, OH^- is shown in Fig. 2. When tested with standards under the protection of ascorbic acid it gave very good recoveries (> 85%) for all estrogens including the labile ones. When using it with the same amounts of estrogens added to urine the recovery of the labile estrogens and especially 2-hydroxyestrogens was much lower. Also several extraneous peaks interfered with the estrogen peaks in gas chromatography with the SE-30 capillary column. It was obvious that the purer the samples applied to the anion exchanger the better the recoveries obtained. For this reason and for the purpose of obtaining purer chromatograms, an appropriate prepurification step prior to the DEAE-Sephadex A-25,OH^- anion exchanger was necessary. Since estrogens behave as weak organic acids during the anion exchange chromatography, the main interfering impurities were other organic acids and phenols. Because most of them are stronger acids than the estrogens the DEAE-Sephadex A-25 gel in the acetate form was found very useful for their removal. It is a weak anion exchanger which does not sorb the estrogens but which is capable of sorbing other stronger organic acids and probably other stronger phenols. The recoveries of all estrogens were essentially quantitative when the gel was used as shown in Fig. 2.

After such prepurification the recoveries of the labile estrogens in the final anion exchange DEAE-Sephadex A-25,OH^- were improved and the purity of

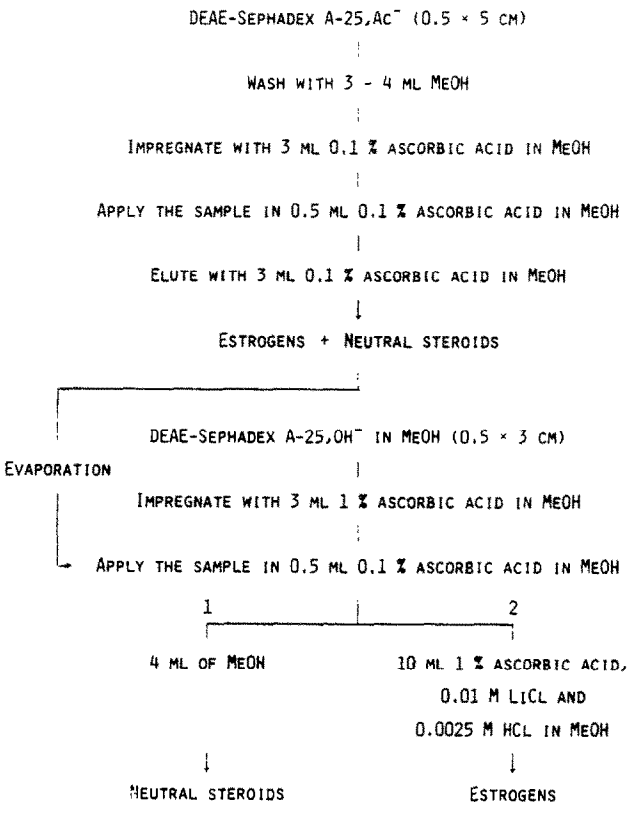

Fig. 2. Flow-diagram of the two-step anion exchange chromatography on DEAE-Sephadex A-25,Ac^- and DEAE-Sephadex A-25,OH^- form. Glassware was silanized.

the final chromatographic eluate was remarkable. These results are summarized in Table 2.

DISCUSSION

From the above reported results it is obvious that in the case ofion exchange chromatography of stable estrogens all the three anion exchangers tested perform equally well. The recoveries of these estrogens are excellent even without the use of ascorbic acid.

The difficulties arise when labile estrogens must be chromatographed through ion exchange chromatography. Some of them, such as the 2-hydroxyestrogens, undergo rapid autoxidative decomposition. Others undergo conversions to related forms, e.g. 16α-hydroxyestrone is frequently converted to 16-oxoestradiol. To avert these problems ascorbic acid must be employed. Another factor influencing the preservation of the labile estrogens is the duration of the ion exchange chromatography. Consequently small columns give better recoveries than large columns. Since (0.55 × 3 cm) columns in short Pasteur pipettes are sufficient for 20–25 ml of urine we adopted their use. The purity of the sample is also very important because the purer the sample applied on the anion exchanger the better are the recoveries of the labile estrogens. This fact was very obvious when the same estrogens were chromatographed alone and together with a urinary extract. Therefore the acetate form of the corresponding anion exchanger was used as a prepurification step. By this way the recoveries using the ion exchanger in the

Table 2. Effect of the sample purity on the recoveries of estrogens in anion-exchange chromatography with DEAE-Sephadex A-25,OH^- (column size: 0.5 × 3 cm), glassware was silanized

Amounts added	1–3 μg	1–3 μg	1–3 μg
Prepurification (DEAE-Sephadex A-25,Ac^-)	No	No	Yes
Urine	No	Yes	Yes
Estrone	90–95%	~90%	~90%
2-Hydroxyestrone	90%	30–35%	50–60%
16α-Hydroxyestrone	85–90%	60–70%	70–80%
16-Oxoestradiol	85–90%	60–70%	70–80%
Estriol	90–95%	~90%	~90%
2-Hydroxyestriol	90%	30–35%	50–60%

OH^- form were increased and the final chromatogram obtained with capillary gas chromatography did not show interfering peaks. However, the acetate form of the AG1-X2 resin partially sorbs some estrogens and thus it is not suitable as a prepurification step.

The results obtained for the three ion exchangers tested demonstrated that the diethylamino ethyl (DEAE)-Sephadex gel gives the best results. The AG1-X2 resin in the bicarbonate form was difficult to impregnate with ascorbic acid. The impregnation with ascorbic acid solution (neutralized with 1 N NaOH) at pH 7 permitted the impregnation of large (1 × 10 cm) columns and this is in accordance with the results of Cohen *et al.*[11]. When columns of (0.5 × 3 cm) size were impregnated in the same way the estrogens were eluted together with the neutral steroids. There seems to be no good theoretical explanation for this. The employment of DTT partially solved this problem but the recoveries of the 2-hydroxyestrogens were not satisfactory and the reproducibility was poor. The Bio-Rex 5 resin had the disadvantage of high nonspecific adsorption of the ring D-ketolic estrogens while the recoveries of the 2-hydroxyestrogens were the same as with the AG1-X2 resin. However, the impregnation with ascorbic acid was more satisfactory with Bio-Rex 5 than with AG1-X2 because the resin could be used in the OH^- form.

The DEAE-Sephadex gel gave the most satisfactory results both with standards alone and with standards added to urinary extracts. The impregnation with ascorbic acid could be achieved easier and the nonspecific adsorption of the ring D-ketolic estrogens was not so profound as in the Bio-Rex 5 resin. The DEAE-Sephadex, Ac^- column is always necessary prior to the DEAE-Sephadex OH^- anion exchange chromatography in order to remove stronger organic acids than estrogens. Since in the development of a method for the whole estrogen profile, group separation of the estrogens is needed, the DEAE-Sephadex OH^- anion exchange chromatography is better done separately for the different groups obtained because the higher purity of the extract results in better recoveries from the column.

Two other anion exchangers have already been used [20–21] for the separation of the estrogens from neutral steroids, the DEAP-LH-20 and the triethylaminohydroxypropyl-hydroxyalkyl (TEAPHA) Sephadex LH-20 [20–21]. Since they are not commercially available we did not study their performance with the labile estrogens.

It is concluded that the two-step anion exchange chromatography, first on the DEAE-Sephadex A-25 gel in the acetate form and then on the same gel in the hydroxyl form, is a suitable purification step if the whole estrogen profile including the labile estrogens is to be assayed.

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