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INFLUENCE OF COLUMN LENGTH AND PORE SIZE ON HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY OF ESTROGEN AND PROGESTIN RECEPTORS*

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SUMMARY

A high-performance liquid chromatographic (HPLC) procedure has been evaluated to establish a routine test in the clinical laboratory for measuring the profiles of estrogen and progestin receptor isoforms in human breast and endometrial tumors. This procedure will be used to determine if there is a relationship between particular isoform profiles and response to various endocrine therapies. Evaluation of various HPLC modes has shown that high-performance ion-exchange chromatography (HPIEC) with silica-based anion exchangers offers a promising approach. In this paper, we have compared HPIEC columns of different lengths (10 and 25 cm) and pore sizes (300, 500 and 1000 Å) in order to obtain an optimal separation procedure. Because of receptor lability, all investigations were performed at 4°C. The mobile phase consisted of 10–500 m*M* phosphate buffer, supplemented with the stabilizing agent, sodium molybdate at pH 7.4. Recoveries from each of the columns were between 70–100%. The length of the column did not influence significantly the retention time and salt concentration required for elution of receptor proteins. However, pore sizes appeared to alter these parameters. With a larger pore size (1000 Å), the retention of proteins was lower (elution with 50 m*M* phosphate) than that observed with the 500-Å pore size column (elution with 100 m*M* phosphate) or of the 300-Å pore size column (elution with 150 m*M* phosphate). Based solely on recovery patterns and peak shape, we conclude that separation of receptor isoforms on a 1000-Å, 25-cm column is best suited for clinical analysis.

INTRODUCTION

Surgical ablative and additive endocrine treatments are effective in about one third of all patients with advanced breast cancer [1]. Analyses of estrogen and progestin receptors in breast cancer tissue have been used routinely to predict endocrine responsiveness [2]. The presence of both estrogen and progestin re-

*This paper is dedicated to S.S. Jung's father, W.Y. Jung, MD, on the occasion of his 63rd birthday.

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ceptors in breast tumor biopsies correlated with objective remissions in 75–80% of patients given endocrine manipulation [2,3]. Moreover, the presence of the progestin receptors in a biospy of endometrial carcinoma correlates with response to progestin therapy [4] and improved prognosis [5]. The molecular basis of unresponsiveness by breast tumors with positive receptors has not been ascertained although receptor defects have been proposed [6,7].

Recently, our laboratory demonstrated that estrogen as well as progestin receptors exhibit polymorphism in that they often consist of several species, based on size, shape, surface charge and hydrophobic properties. These molecular species are termed isoforms [8]. The origin of the multiple forms of receptors (isoforms) and their physiological significance are major foci of investigation by our laboratory [3,8,9]. Although we have evidence that certain isoforms have biological significance [10,11], some receptor species must be due to proteolytic cleavage during homogenization, prolonged incubation and/or overnight centrifugation [12]. To ovecome these problems, we developed a high-performance liquid chromatographic (HPLC) method based on properties of size, shape, surface charge and hydrophobicity [9,10,11,13]. Among these, high-performance ion-exchange chromatography (HPIEC) is one of the most effective HPLC modes for separation of receptor isoforms in terms of resolution and recovery [14, 15].

There are many parameters to evaluate in order to utilize HPIEC including the exchanger in the stationary phase, buffer, gradient type and shape as well as pore size and length of column. Silica has been widely used for the separation of proteins because of its favorable pore structure and variability in surface composition [16, 17]. The first commercially available, weak anion-exchange columns with porosities compatible with proteins were introduced by SynChrom (Linden, IN, U.S.A.) in 1978, followed by those of Pharmacia (Piscataway, NJ, U.S.A.) and of Toya Soda (Japan) in 1982. In this report we utilize HPLC to compare experimental weak anion exchange made on a porous silica back bone of different pore sizes and lengths from CliniChrom Labs. (Lafayette, IN, U.S.A.). Separation of isoforms was accomplished using HPLC and radioactively labeled steroids as ligands [9, 13]. The objective of this study was to establish an HPLC procedure which could be used routinely in the clinical laboratory to measure the profiles of estrogen and progestin receptor isoforms in human breast and endometrial tumors. With a standardized method, we subsequently determined the relationship between a particular isoform profile and response to various endocrine therapies. In this paper, we report an optimal procedure for the identification of receptor isoforms using comparatively weak anion-exchange HPLC columns of different lengths (10 and 25 cm) and pore sizes (300, 500 and 1000 Å).

EXPERIMENTAL

Materials

All reagents were of analytical grade. The ligands, [2,4,6,7-^{3}H]estradiol-17β, and [17α-methyl-^{3}H]R5020 (promegestone) were purchased from DuPont/NEN Products (Boston, MA, U.S.A.). Unlabeled diethylstilbesterol (DES)

and R5020 were used as competitors for estrogen and progestin receptors, respectively. Disodium ethylenediaminetetraacetic acid (EDTA) and glycerol were obtained from Fisher Scientific (Cincinnati, OH, U.S.A.). Norit-A, dextran T-70, sodium molybdate and dithiothreitol (DTT) were obtained from Sigma (St. Louis, MO, U.S.A.).

Because of the possible variability in receptor isoforms in different individual human tumors, we pooled numerous biopsies known to contain both estrogen and progestin receptors. These pooled tissues were prepared in a manner similar to that we described earlier [18] to insure a homogenous reference powder for all experiments described in this report. The specific estrogen binding capacity of cytosol from the unfractionated preparation was 129 ± 13 fmol per mg cytosol protein and the K_d value was $1.9 \pm 0.5 \cdot 10^{-10}$ *M*. The specific progestin receptor content was 213 ± 11 fmol per mg cytosol protein with a K_d value of $1.3 \pm 0.5 \cdot 10^{-10}$ *M*.

Preparation of the cytosol

Pooled human breast cancer tissues were homogenized at 1:2.5 (w/v) in P_{10}EDG buffer designated buffer A [10 m*M* potassium phosphate, pH 7.4, containing 1.5 m*M* EDTA, 1 m*M* DTT, 10% (v/v) glycerol] and 10 m*M* sodium molybdate using a Brinkman® polytron homogenizer (Westbury, NY, U.S.A.) for two 5-s bursts. The homogenate was then centrifuged at 200 340 *g* using a Beckman (Palo Alto, CA, U.S.A.) TL-100 tabletop ultracentrifuge with TLA 100.2 rotor for 15 min. All procedures were performd at 0–4 °C. The supernatant was obtained as the cytosol.

Receptor–ligand binding reaction

The cytosols containing receptors were incubated with 4 n*M* of either [^{3}H]estradiol or [^{3}H]R5020 for 16 h at 0 °C in the presence (non-specific binding) or absence (total binding) of a 200-fold molar excess of the appropriate unlabeled competitor, DES and R5020, respectively. The incubations were terminated by removing unbound steroid with a pellet derived from an equal volume of a 1% dextran-coated charcoal suspension (1% charcoal, 0.5% dextran) in buffer A. After the mixture reacted for 5 min, it was centrifuged at 2000 *g* for 10 min. The supernatant obtained represented a mixture of radioligand-associated receptor isoforms which were used for HPIEC.

Protein determination

Protein concentrations were determined by the method of Bradford [19] using Bio-Rad reagent (Bio-Rad Labs., Richmond, CA, U.S.A.) and bovine serum albumin as the standard.

High-performance ion-exchange chromatography

Aliquots (200 μl) of the labeled receptors were cleared of unbound ligand and applied to various anion-exchange columns with an Altex 322 (Beckman, Berkeley, CA, U.S.A.) chromatographic system equipped with several flow-through detectors as described earlier [10]. The columns were of different lengths (10 and 25 cm) and pore sizes (300, 500 and 1000 Å) consisting of a silica-based,

anion-exchange stationary phase (CliniChrom Labs.). All chromatographic separations were performed in a cold room at 0–4 °C. Buffers were filtered through 0.45-μm Millipore filters (Bedford, MA, U.S.A.) prior to use. Each column was precycled with low-ionic-strength phosphate buffer (buffer A). Buffer B was the same as buffer A except that the phosphate concentration was 500 m*M*.

The flow-rate of the mobile phase for HPIEC separation was 1 ml/min. The gradient elution was programmed as follows using a two-pump solvent delivery system and controller (Beckman, Model 420). The sample was injected at $t=0.5$ min with 100% buffer A. The column was washed for 9.5 min with 100% buffer A after which the salt gradient was initiated. It approached 50% buffer B in the next 25 min. At $t=35$ min, the mobile phase was changed to increase to 100% buffer B in the next 12 min. At $t=47$ min, it was changed to 100% buffer A for an additional 8 min to return to the original 10 m*M* phosphate concentration. The total elution time was 55 min.

The phosphate concentration was monitored with an in-line Bio-Rad conductivity monitor, which graphed results on a Kipp & Zonen BD 41 chart recorder. Fractions were collected with a Buchler fraction collector (Fort Lee, NJ, U.S.A.). [^{3}H]Ligand–receptor complexes were measured by counting radioactivity in column fractions with a Model 1801 liquid scintillation counter (Beckman). Specific binding capacity of receptors was expressed as fmol of steroid bound per mg of cytosol protein.

RESULTS AND DISCUSSION

Fig. 1 illustrates a typical HPIEC profile of estrogen receptors in the presence of molybdate using one of the ion-exchange columns to demonstrate the specificity of the reaction and the phosphate gradient. It should be noted that inclusion of sodium molybdate in homogenization buffer brings about a relative stabilization of steroid receptors [20] resulting in higher molecular forms as we reported

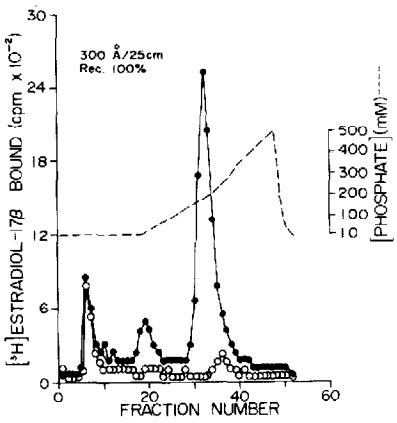

Fig. 1. Representative HPIEC profile of estrogen receptors in human breast cancer. The column was a CliniChrom anion exchanger; 300 Å pore size, 25 cm×4.6 mm I.D.; flow-rate, 1 ml/min; buffer, potassium phosphate, 55 min gradient from 10 to 500 m*M* concentration.

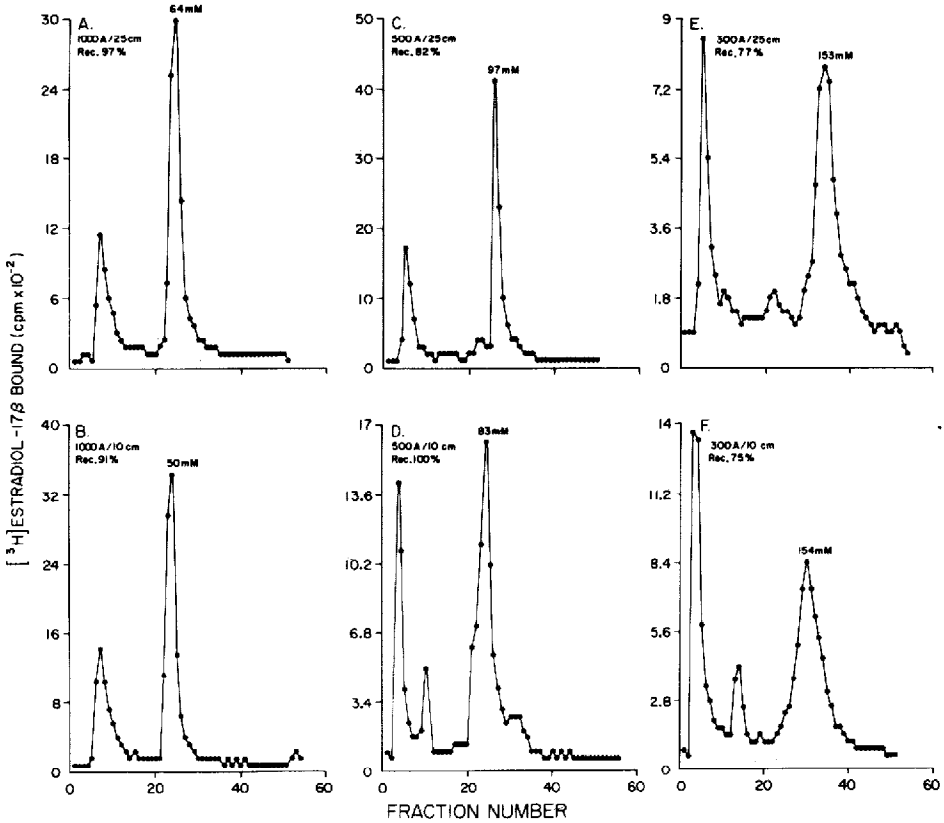

Fig. 2. Representative elution profiles of estrogen receptors separated by various weak anion-exchange columns of different silica pore sizes and lengths. (A) 1000 Å, 25 cm×4.6 mm I.D.; (B) 1000 Å, 10 cm×4.6 mm I.D.; (C) 500 Å, 25 cm×4.6 mm I.D.; (D) 500 Å, 10 cm×4.6 mm I.D.; (E) 300 Å, 25 cm×4.6 mm I.D.; (F) 300 Å, 10 cm×4.6 mm I.D. Conditions were identical to those described in Fig. 1.

earlier using high-performance size-exclusion chromatography [21]. As seen in Fig. 1 there was a predominant estrogen receptor isoform separating at a retention time of 32 min. Note that the ligand binding profile of the cytosol previously incubated with [^{3}H]estradiol and DES showed specific steroid binding only by this component. The shape of the gradient described in Experimental is also shown in Fig. 1. For convenience of illustration, only the profiles of total binding are presented in Figs. 2 and 3. The peaks identified with molar concentration of phosphate represent the elution positions of the principal receptor isoform. The other peaks represent either non-specific binding or minor receptor isoforms.

The elution peak on both 10- and 25-cm columns with the same pore size showed no significant difference but there was a tendency toward a slight broadening of the peak on the 10-cm column for both estrogen and progestin receptors (Figs. 2 and 3). Cooper [22] demonstrated that long narrow columns usually give considerably less resolution than a column with a higher diameter-to-length ratio on DEAE-cellulose exchange media. We do not know the reason of contradiction between our analyses and those of Cooper [22].

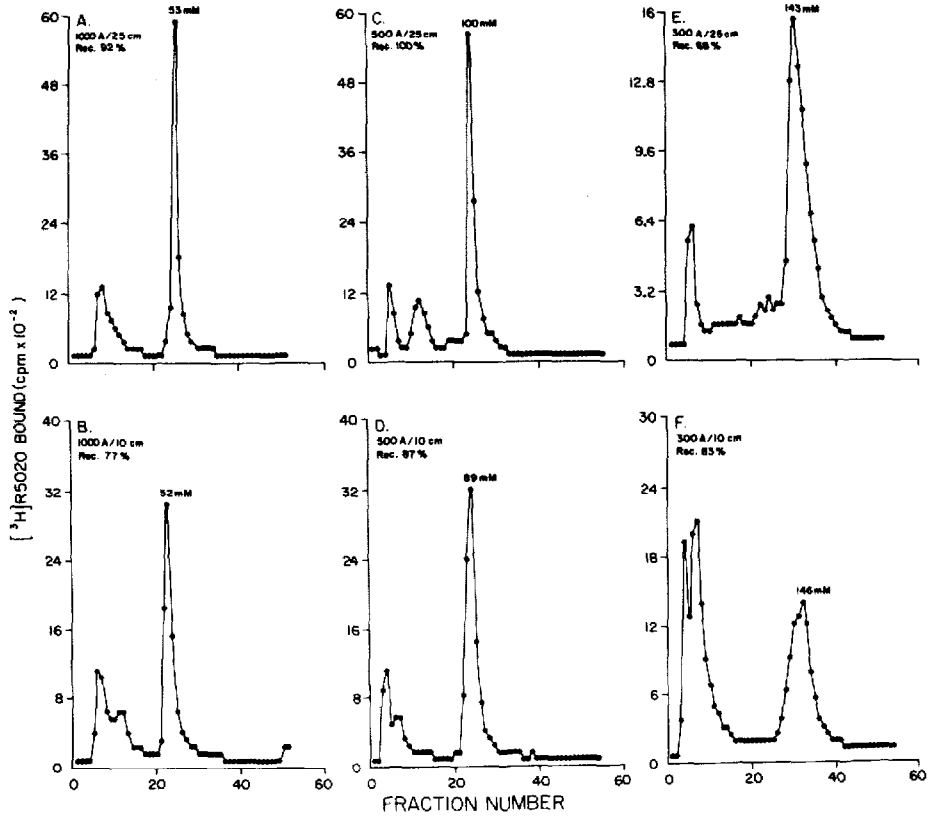

Fig. 3. Representative elution profiles of progestin receptors separated by various weak anion-exchange columns of different silica pore sizes and lengths. (A) 1000 Å, 25 cm×4.6 mm I.D.; (B) 1000 Å, 10 cm×4.6 mm I.D.; (C) 500 Å, 25 cm×4.6 mm I.D.; (D) 500 Å, 10 cm×4.6 mm I.D.; (E) 300 Å, 25 cm×4.6 mm I.D.; (F) 300 Å, 10 cm×4.6 mm I.D. Conditions were identical to those described in Fig. 1.

As shown in Table I, the principal estrogen receptor isoform was eluted from the 25-cm columns with 150 m*M* phosphate using 300 Å, 102 m*M* using 500 Å, and 53 m*M* using 100 Å pore size. Virtually the same results were observed using the 10-cm column. Here the estrogen receptor isoform eluted at 153 m*M* phosphate with the 300 Å, 94 m*M* with the 500 Å and 50 m*M* with 1000 Å pore size. The principal progestin receptor isoform eluted at concentrations of phosphate very similar to that used for the estrogen receptor. As shown in Table II, using the 25-cm column, the progestin receptor eluted at 141 m*M* at 300 Å, 103 m*M* at 500 Å, and 48 m*M* at 1000 Å pore size. The elution profiles using the 10-cm columns showed the isoform released with 144 m*M* at 300 Å, 84 m*M* at 500 Å, and 47 m*M* at 1000 Å pore size.

As shown in Tables I and II when elution data were expressed as a function of retention time given in minutes, there was greater agreement between the 10- and 25-cm columns. These results clearly demonstrate that both receptor isoforms eluted from either column length with a retention time that increased as a

TABLE I

ELUTION OF ESTROGEN RECEPTORS IN CYTOSOL FROM HUMAN BREAST CANCER USING HPIEC

Collective results are shown for the elution characteristics of the receptors using different pore sizes and column lengths. Data (mean ± S.D.) represent the phosphate concentrations at which the receptors were eluted and the retention times. The recovery of specific steroid-binding activity is expressed in percent of receptor applied to the column.

Pore size (Å)	Column of 25 cm				Column of 10 cm			
	n	Phosphate concentration (m*M*)	Retention time (min)	Recovery (%)	*n*	Phosphate concentration (m*M*)	Retention time (min)	Recovery (%)
300	5	150 ± 3	33 ± 2	71 ± 9	5	153 ± 3	32 ± 1	84 ± 9
500	5	102 ± 5	28 ± 1	78 ± 11	6	94 ± 13	26 ± 1	78 ± 19
1000	7	53 ± 4	25 ± 1	91 ± 16	5	50 ± 2	24 ± 1	85 ± 14

TABLE II

ELUTION OF PROGESTIN RECEPTORS IN CYTOSOL FROM HUMAN BREAST CANCER USING HPIEC

Collective results are shown for the elution characteristics of the receptors using different pore sizes and column lengths. Data (mean ± S.D.) represent the phosphate concentrations at which the receptors were eluted and the retention times. The recovery of specific steroid-binding activity is expressed in percent of receptor applied to the column.

Pore size (Å)	Column of 25 cm				Column of 10 cm			
	n	Phosphate concentration (m*M*)	Retention time (min)	Recovery (%)	*n*	Phosphate concentration (m*M*)	Retention time (min)	Recovery (%)
300	6	141 ± 3	31 ± 1	75 ± 20	6	144 ± 8	31 ± 1	86 ± 9
500	5	103 ± 16	27 ± 1	72 ± 17	5	84 ± 21	26 ± 1	89 ± 8
1000	5	48 ± 4	25 ± 1	84 ± 12	5	47 ± 9	24 ± 2	76 ± 10

function of decreasing pore size. A relationship between pore size and elution of proteins has been observed previously [9, 17, 18]. Unger [16] suggested that a stationary phase consisting of silica-based material may change the porosity of the packed bed thus influencing the retention time and separation of proteins. Gooding and Schmuck [17] reported that the 300 Å pore size gave a superior resolution of proteins smaller than 100 000 in molecular mass such as albumin but the 1000 Å pore was more appropriate for higher-molecular-mass proteins in excess of 200 000 such as catalase. Molecular mass for estrogen and progestin receptors is greater than 300 000.

To evaluate the reproducibility of these separations, HPIEC was performed on both estrogen and progestin receptors using a single reference breast cancer cytosol in a single experiment. Data shown in Table III illustrate the reproducibility of the elution position of isoforms of estrogen and progestin receptor using the

TABLE III

REPRODUCIBILITY OF ELUTION POSITION OF STEROID HORMONE RECEPTORS USING AN EXPERIMENTAL 1000-Å, 25-cm COLUMN AND HPIEC

Collective results are shown for the elution characteristics of the receptors using different pore sizes and column lengths. Data (mean ± S.D., $n=5$) represent the phosphate concentrations at which the receptors were eluted and the retention times. The recovery of specific steroid-binding activity is expressed in percent of receptor applied to the column. The coefficient of variation of elution position is shown for each type of receptor

Receptor	Phosphate concentration (m*M*)	Retention time (min)	Coefficient of variation (%)	Recovery (%)	Coefficient of variation (%)
Estrogen	45 ± 1	25 ± 1	2.2	99 ± 2	2.0
Progestin	46 ± 2	25 ± 1	4.3	99 ± 1	1.0

TABLE IV

REPRODUCIBILITY OF ELUTION POSITION OF STEROID HORMONE RECEPTORS USING AN EXPERIMENTAL 300-Å, 25-cm COLUMN AND HPIEC

Collective results are shown for the elution characteristics of the receptors using different pore sizes and column lengths. Data (mean ± S.D., $n=4$) represent the phosphate concentrations at which the receptors were eluted and the retention times. The recovery of specific steroid-binding activity is expressed in percent of receptor applied to the column. The coefficient of variation of elution position is shown for each type of receptor.

Receptor	Phosphate concentration (m*M*)	Retention time (min)	Coefficient of variation (%)	Recovery (%)	Coefficient of variation (%)
Estrogen	151 ± 10	32 ± 1	4.6	86 ± 4	4.7
Progestin	144 ± 4	32 ± 1	2.8	83 ± 4	4.8

25-cm, 1000-Å column. The estrogen receptor eluted at 45 ± 1 m*M* phosphate while the progestin receptor eluted at 46 ± 2 m*M* phosphate when five identical runs were performed in a single day. The coefficient of variation of the elution position was 2.2% for the estrogen receptor and 4.3% for the progestin receptor. Note in both cases the retention times were 25 ± 1 min with recoveries of 99% of the activity.

A similar experiment of the reproducibility of the elution position of both estrogen and progestin receptors were performed on the 25-cm column using the 300 Å pore size. As shown in Table IV, the estrogen receptor isoform eluted at 151 ± 10 m*M* phosphate while the progestin receptor eluted at 144 ± 4 m*M* phosphate. The retention times were identical, 32 ± 1 min, with recoveries above 80%. The coefficient of variation of the estrogen receptor was 4.6% while that of the progestin receptor was 2.8%. These data clearly indicate that HPIEC using a variety of pore sizes may be used to study the structural characteristics of estrogen and progestin receptors in a highly efficient and rapid fashion.

CONCLUSION

In summary, this study demonstrates that estrogen and progestin receptors isolated in the presence of the stabilizer, sodium molybdate, elute as a principal charge isoform using silica-based, weak anion-exchange stationary phases. The 25-cm column appears to be superior to the 10-cm column primarily due to the fact that although there was no alteration in elution behaviour with length, there was an increase in the capacity of the column. The length of the column did not alter the elution position of either estrogen or progestin receptor. However, pore size ranging from 300 to 1000 Å clearly influenced the retention time. Retention of either estrogen or progestin receptor isoforms increased with decreasing pore size. We believe that a greater number of contact points between the receptors and the stationary phase were available on the 300-Å column. The 1000-Å anion-exchange column may have fewer contact points. This will allow for earlier release of receptors from the 1000-Å column when the increasing salt gradient is initiated. These data suggest that both the estrogen and the progestin receptor exist as highly charged receptor species which may associate readily with weak anion exchanger. Based on the results of this study, we recommend that the 25-cm column with 1000-Å silica is a highly suitable mode of HPLC for the routine separation of estrogen and progestin receptors. This conclusion is based upon the high recoveries observed and the superior performance characteristics indicating good reproducibility with coefficients of variation of less than 5%.

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REFERENCES

1 C.G. Kardinal, in W.L. Donegan and J.S. Spratt (Editors), Cancer of the Breast, W.B. Saunders, Philadelphia, PA, in press.
2 Anonymous, Cancer, 46 (1980) 2759.
3 J.L. Wittliff, Cancer, 56 (1984) 630.
4 C.E. Erlich, P.C. Young and R.E. Cleary, Am. J. Obstet. Gynecol., 141 (1981) 539.
5 W.T. Creasman, J.T.Soper, K.S. McCarty, Jr., K.S. McCarty, Sr., W. Hinshaw and D.L. Clarke-Pearson, Am. J. Obstet. Gynecol., 151 (1985) 922.
6 J.L. Wittliff and E.D. Savlov, in W.L. McGuire, P.P. Carbone and E.P. Vollmer (Editors), Estrogen Receptors in Human Breast Cancer, Raven Press, New York, 1975, p. 73.
7 J.L. Wittliff, W.M. Lewko, D.C. Park, T.E. Kute, D.T. Baker, Jr. and L.N. Kane, in W.L. McGuire (Editor), Hormones, Receptors and Breast Cancer, Raven Pess, New York, 1978, p. 325.

8 J.L. Wittliff, P.A. Feldhoff, A. Fuchs and R.D. Wiehle, in R. Soto, A.F. DeNicola and J.A. Blaquier (Editors), Physiopathology of Endocrine Diseases and Mechanisms of Hormone Action, Alan R. Liss, New York, 1981, p. 375.
9 J.L. Wittliff, LC·GC, Mag. Liq. Gas Chromatogr., 4 (1986) 1092.
10 L.A. van der Walt and J.L. Wittliff, J. Steroid Biochem., 24 (1986) 377.
11 J.L. Wittliff, N.A. Shahabi, S.M. Hyder, L.A. van der Walt, L. Myatt, D.M. Boyle and Y.J. He, in J. L'Italien (Editor), Modern Methods in Protein Chemistry, Plenum Press, New York, 1987, in press.
12 M.R. Sherman, L.A. Pickering, H.M. Rollwagen and L.K. Miller, Fed. Proc., Fed. Am. Soc. Exp. Biol., 37 (1978) 167.
13 J.L. Wittliff and R.D. Wiehle, in V.P. Hollander (Editor), Hormonally Responsive Tumors, Academic Press, New York, 1985, p. 383.
14 R.D. Wiehle and J.L. Wittliff, J. Chromatogr., 297 (1984) 313.
15 D.M. Boyle, R.D. Wiehle, N.A. Shahabi and J.L. Wittliff, J. Chromatogr., 327 (1985) 369.
16 K. Unger, Porous Silica, Elsevier, Amsterdam, 1979, p. 11.
17 K.M. Gooding and M.N. Schmuck, J. Chromatogr., 327 (1985) 139.
18 J.L. Wittliff, J.L. Durant and B. Fisher, in R. Soto, A.F. DeNicola and J.A. Blaquier (Editors), Physiopathology of Endocrine Diseases and Mechanisms of Hormone Action, Alan R. Liss, New York, 1981, p. 397.
19 M.M. Bradford, Anal. Biochem., 72 (1976) 248.
20 M.K. Dahmer, P.R. Housley and W.B. Pratt, Ann. Rev. Physiol., 46 (1984) 67.
21 R.D. Wiehle, G.E. Hofmann, A. Fuchs and J.L. Wittliff, J. Chromatogr., 307 (1984) 39.
22 T.G. Cooper, The Tools of Biochemistry, Wiley, New York, 1977, p. 150.