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Supercritical fluid chromatography of selected oestrogens

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Abstract: The separation of selected oestrogens (oestrone, equilin, α-oestradiol, β-oestradiol and *d*-equilenin) using capillary supercritical fluid chromatography (SFC) was studied. Three different stationary pahses (SB-methyl-100, SB-biphenyl-30 and SB-cyanopropyl-50) were studied for the separation of the compounds. A baseline separation of the oestrogens was achieved on a SB-cyanopropyl-50 column using a carbon dioxide density gradient at an oven temperature of 73°C. Typical analysis time on a 7 m column was 21 min. Retention times of each oestrogen decreased with an increase in either mobile phase density or oven temperature. Accuracy and precision of the SFC method were in the 1–5.5% range. The SFC method was applied to three different dosage forms containing oestrogens.

Keywords: *Capillary SFC; oestrogens; SB-cyanopropyl 50; carbon dioxide density gradient.*

Introduction

Supercritical fluid chromatography (SFC) is a rapidly developing analytical technique that generates a high number of theoretical plates and has the advantage of using the universal flame ionization detector (FID) with capillary columns [1]. When working with pharmaceuticals in a complex mixture, FID is advantageous, because one does not have to work with a compromised absorption maximum for the analytes in a given sample.

The authors' laboratory has been interested in applying SFC as an analytical technique to pharmaceutically important compounds. There are some literature reports on the use of supercritical fluid chromatography in the analysis of pharmaceuticals such as steroids, cannabinoids, barbiturates and antibiotics [2–6]. The SFC separation of non-steroidal anti-inflammatory agents on biphenyl and cyanopropyl stationary phases in capillary columns has recently been reported [7]. In a continuation of these studies, the use of SFC in the analysis of oestrogens has been investigated. Among the analytical methods reported in the literature for oestrogens are TLC [8–12], GC [13–17] and LC [18–20]. These techniques were used to separate and, in some cases, quantitate each oestrogen in relatively complex mixtures. If the oestrogens exist as sulphate esters, a simple enzymatic or acid hydrolysis is usually used to convert the compounds into the 'free' oestrogens, which can be extracted into an organic phase and then analysed. The TLC assays, sample preparation and plate development are time-consuming steps. In GC assays, the free oestrogens must be chemically derivatized, to form trimethylsilyl, methoxamine-trimethylsilyl, or oxime-trimethylsilylesters derivatives in order to achieve successful chromatography. In LC studies, dansyl or other fluorescent derivatives must be formed with the oestrogens to achieve the desired sensitivity.

Little, if any, information is available on the use of supercritical fluid chromatography in the analysis of oestrogens. Chang and Lee have reported on the separation of selected oestrogens using a liquid polysiloxane capillary column [21]. In another report, David and Novotny reported the derivatization of oestrogens with dimethylthiophosphinic chloride and SFC separation on a SE-33 capillary column using a phosphorous-selective detector [22].

In this paper, the results of the application of capillary column SFC, with flame ionization detection, to the separation of selected oestrogens are presented. The SFC method is simple and rapid, the separation of the oestrogens

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studied (oestrone, equilin, α-oestradiol, β-oestradiol and *d*-equilenin) was achieve in 21 min on a 7 m cyanopropyl-50 column using an oven temperature of 73°C and a carbon dioxide density gradient.

Experimental

Materials

SFC grade carbon dioxide was obtained from Scott Specialty Gases (Plumsteadville, PA, USA). Chloroform and methylene chloride were HPLC grade (J.T. Baker, Phillipsburg, NJ, USA). Oestrone, α-oestradiol, β-oestradiol, equilin, *d*-equilenin and methyltestosterone were purchased from Sigma Chemical Company (St Louis, MO, USA). Commercial tablets of Estrace, Lot MJC42, Menrium, Lot 1914 and Estratest, Lot 82944 were obtained from a local pharmacy. Standard solutions were prepared by accurately weighing approximately 5 mg of each oestrogen and dissolving in 5 ml of chloroform to obtain a final concentration of approximately 1 mg ml^{-1}.

Chromatography

The supercritical fluid chromatograph used was a Lee Scientific Model 600D Supercritical Fluid Chromatograph (Salt Lake City, UT, USA) equipped with a pump, oven and flame ionization detector and controlled by a Dell computer (Austin, TX, USA) (ACI 600D, Software version 2.2). Three different stationary phases were utilized: a 5 m × 100 μm i.d. SB-methyl-100 (200 μm o.d. and 0.25 μm film thickness), a 10 m × 50 μm i.d. SB-biphenyl-30 and a 10 m × 50 μm i.d. SB-cyanopropyl-50 (both 195 μm o.d. and 0.25 μm film thickness). All three columns were purchased from Lee Scientific. The following conditions were used for the assay. Pump program: multilinear density program: 6 min hold at an initial density of 0.7000 g m^{-1}, then 0.0064 g ml^{-1} min^{-1} ramp to 0.7500 g ml^{-1}, followed by a 0.0100 g ml^{-1} min^{-1} ramp to 0.08300 g ml^{-1}. Oven temperature: isothermal at 73°C. Column: 7 m × 50 μm SB-cyanopropyl-50. Injection type: time split set at 500 ms. Injection ratio approximately 10 to 1, giving an injection volume of approximately 20 nl. Detector: flame ionization at 375°C. Mobile phase: supercritical fluid grade carbon dioxide. Analysis time: 21 min.

Results and Discussion

Esterified or conjugated oestrogens are described in the United States Pharmacopeia XXII as a mixture of sodium oestrone sulphate and sodium equilin sulphate derived wholly or in part from equine urine, or synthetically from oestrone or equilin [23]. The mixture may also contain other oestrogenic substances of the type excreted by pregnant mares. Esterified or conjugated oestrogens can be easily converted into the 'free' oestrogens using either enzymatic or acid hydrolysis [12, 23]. In this study, the SFC separation of oestrone, equilin, α-oestradiol, β-oestradiol and *d*-equilenin is presented (see Fig. 1). While all of these oestrogens except β-oestradiol are usually present in conjugated or esterified oestrogen dosage forms, β-oestradiol can be found in a single entity dosage form and was included in our study for completeness.

Method development

Three different stationary phases, SB-methyl-100, SB-biphenyl-30 and SB-cyanopropyl-50 were investigated for the separation of these oestrogens. The solvating power of carbon dioxide can be changed by manipulating the pressure or density of the supercritical fluid. At any given temperature, the increase in pressure results in an increase in density even though the relationship may not be linear. The SB-methyl-100 column is coated with 100% methyl polysiloxane and is cross-linked for SFC use. Different oven temperatures (60–140°C) and carbon dioxide pressure gradients (3–30 atm min^{-1}, both as linear and step gradients) were investigated. None of the oestrogens could be separated on the methyl column due to poor peak shape and overlapping of peaks.

The SB-biphenyl-30 column is coated with 30% biphenyl and 70% methyl polysiloxane and is cross-linked for SFC use. After investigating different carbon dioxide pressure gradients (2 atm min^{-1}, both linear and step gradients) and oven temperatures (50–150°C), it was observed that at an oven temperature of 120°C and a pressure gradient (initial pressure at 100 atm, 20 atm min^{-1} to 350 atm, hold at 350 atm for 15 min), only the separation of *d*-equilenin from the remaining oestrogens was achieved. The retention times were approximately 21 min for all the other oestrogens and 26 min for *d*-equilenin. Oven temperatures

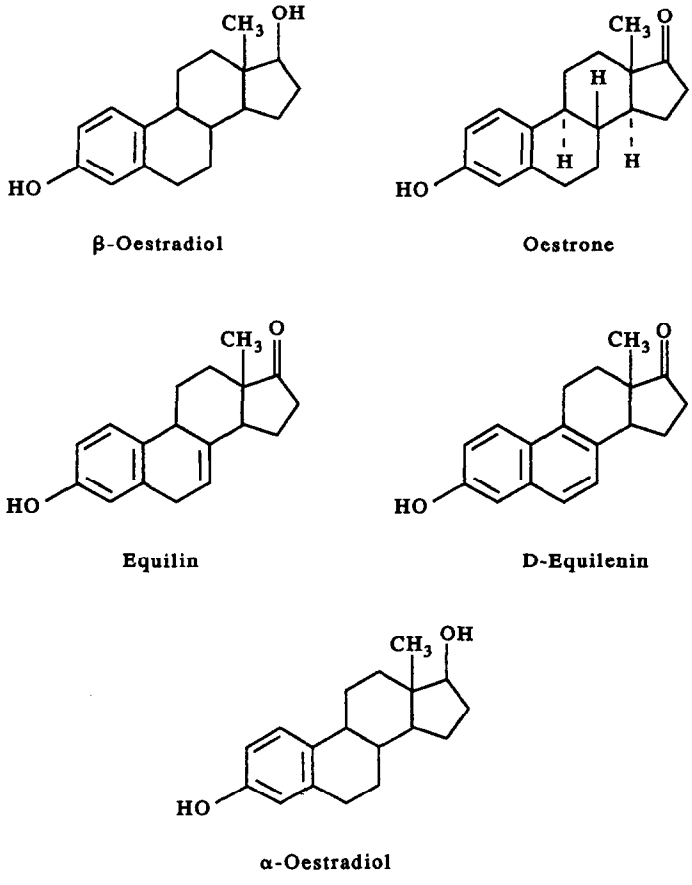

Figure 1
Structural formulae of compounds studied.

below 50°C were not investigated due to excessive baseline noise and drift and extremely broad analyte peaks.

The SB-cyanopropyl-50 column is coated with 50% cyanopropyl and 50% methyl polysiloxane and is considered to be the most polar column among the commercially available SFC columns. The separation of all the oestrogens was achieved on the 10 m column within 56 min. To further reduce the analysis time, the column was shortened to 7 m and the effects of oven temperature and carbon dioxide density were studied on the retention of the oestrogens. It was observed that retention of all analytes decreased with an increase in either density or temperature. Table 1 lists retention times for each oestrogen at the different oven temperatures and densities. A density gradient as described above in Experimental section was developed from the tabular data. To further maximize the separation, the effect of oven temperature on the carbon dioxide gradient was studied. It was observed that there was a log–linear relationship between retention time and oven temperature for all analytes ($r > 0.999$, $n = 8$). At 50°C, even though a separation was obtained, the analyte peaks were very broad (tailing factors more than 2 for each analyte) and the total analysis time was 38 min. At 70°C, all five oestrogens were again separated and peak shapes were much improved compared to 50°C. Then, by finely tuning the oven temperature to 73°C, a baseline separation of all the oestrogens was achieved within 21 min on the cyanopropyl column as shown in Fig. 2.

The separation of the oestrogen mixture was also investigated on a SB-smectic (liquid crystalline polysiloxane) column using a modification of the method reported by Chang and Lee [21]. By increasing the oven temperature to 130°C, it was possible to achieve adequate separation of the oestrogens. The retention times obtained were 25.1, 25.7, 30.3,

Table 1
Effects of carbon dioxide density and oven temperature on oestrogen retention times using a 7 m SB-cyanopropyl 50 capillary column

Compound*	Carbon dioxide density (g ml^{-1})	Oven temperature (°C) 50	70	90	110	130
ES		—†	—†	—†	49.5‡	21.7
EQ		—†	—†	—†	57.4	23.2
αET	0.45	—†	—†	—†	64.7	24.3
βET		—†	—†	—†	64.7	25.9
dEL		—†	—†	—†	104.1	41.7
ES		—†	—†	35.9	14.2	6.7
EQ		—†	—†	41.8	16.2	7.4
αET	0.55	—†	—†	45.0	16.2	7.4
βET		—†	—†	51.3	16.2	7.9
dEL		—†	—†	76.6	27.7	11.9
ES		39.2	30.7	12.0	4.9	2.5
EQ		46.5	35.9	14.8	5.5	2.5
αET	0.65	61.2	41.8	14.9	6.0	2.5
βET		69.4	47.4	16.6	6.0	2.5
dEL		88.2	66.3	23.0	8.7	3.7
ES		36.0	8.8	4.0	—§	—§
EQ		42.5	11.1	4.5	—§	—§
αET	0.75	55.8	11.6	4.5	—§	—§
βET		63.1	12.8	5.2	—§	—§
dEL		80.0	17.0	7.1	—§	—§
ES		9.5	—‖	—§	—§	—§
EQ		10.9	—‖	—§	—§	—§
αET	0.85	14.0	—‖	—§	—§	—§
βET		15.3	—‖	—§	—§	—§
dEL		18.7	—‖	—§	—§	—§

*ES = oestrone, EQ = equilin, αET = α-oestradiol, βET = β-oestradiol and dEL = *d*-equilenin.
†No elution within 2 h.
‡Retention time (min).
§Not within instrumental capacity to reach density at stated oven temperature.
‖All the analytes coelute at solvent front.

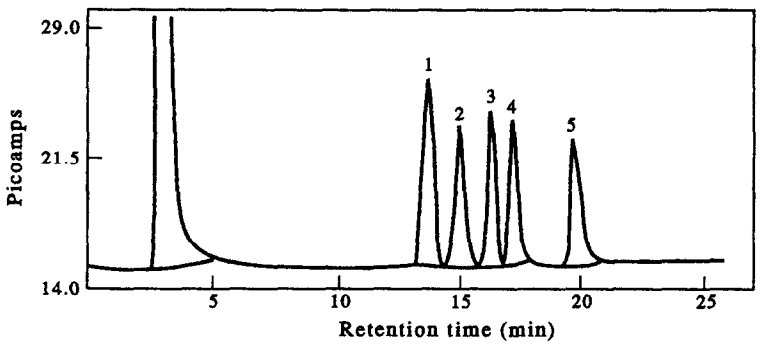

Figure 2
Typical SFC separation of oestrone (1), equilin (2), α-oestradiol (3), β-oestradiol (4), and *d*-equilenin (5) on a SB-cyanopropyl-50 column.

33.0 and 34.1 min for β-oestradiol, α-oestradiol, oestrone, equilin and *d*-equilenin, respectively. The mode of separation on this column is based on molecular geometry rather than mobile phase and stationary phase interactions. The column is relatively unstable chemically and is more expensive than any other commercially available SFC columns.

Thus, the SFC data indicated that the best separation of the oestrogen mixture was obtained on the 7 m cyanopropyl capillary column. The column was considered to be

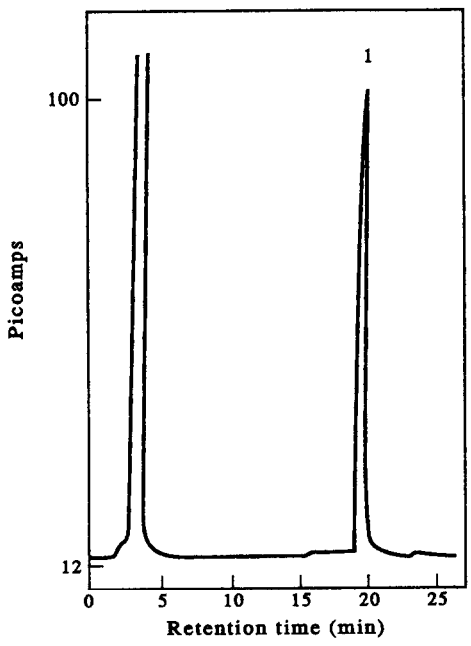

Figure 3
Typical SFC chromatogram of β-oestradiol in a tablet dosage form on a SB-cyanopropyl-50 column.

stable and allowed the baseline separation of the five oestrogens within 21 min.

Validation

Calibration curves for each oestrogen were linear in the 0.5–2.0 mg ml^{-1} range ($r > 0.998$, $n = 3$). Determination of spiked samples of the oestrogen mixtures at 0.75 and 1.5 mg ml^{-1} concentrations indicated that accuracy and precision of the SFC method were within 5.5% (see Table 2). The limit of detection for the oestrogens is in the 125–150 μg ml^{-1} range ($S/N = 3$) and the limit of quantitation is approximately 500 μg ml^{-1} for each oestrogen. The other analytical figures of merit for the oestrogens are listed in Table 3.

Analysis of dosage forms

It was of interest to examine the applicability of the SFC method on the cyanopropyl column to the determination of oestrogens in commercial dosage forms. Three commercial dosage forms were selected for study. They included one in which the oestrogens were conjugated,

Table 2
Accuracy and precision data for selected oestrogens by SFC method

Compound	Conc. added (mg ml^{-1})	Conc. found (mg ml^{-1})*	Per cent error (accuracy)%	RSD%
Oestrone	0.75	0.73 ± 0.008	2.7	1.1
	1.50	1.42 ± 0.030	5.3	2.1
Equilin	0.75	0.75 ± 0.025	0.0	3.3
	1.50	1.46 ± 0.029	2.7	1.9
α-Oestradiol	0.75	0.74 ± 0.028	1.3	3.8
	1.50	1.55 ± 0.044	3.3	2.8
β-Oestradiol	0.75	0.71 ± 0.012	5.3	1.7
	1.50	1.45 ± 0.045	3.3	3.1
d-Equilenin	0.75	0.72 ± 0.025	4.0	3.5
	1.50	1.44 ± 0.060	4.0	4.2

*Mean ± standard deviation based on $n = 3$.

Table 3
Analytical figures of merit for selected oestrogens on a 7 m SB-cyanopropyl-50 column with FID detection and carbon dioxide mobile phase

Compound	Retention time (min)	Regression coefficient (r, $n = 3$)*	Tailing factor†	LOD‡ (ng)	LOD‡ (μg ml^{-1})
Oestrone	12.9	0.9993	1.04	3.0	150
Equilin	14.2	0.9998	1.13	3.5	175
α-Oestradiol	15.5	0.9993	1.12	3.0	150
β-Oestradiol	16.4	0.9997	1.12	2.5	125
d-Equilenin	19.0	0.9986	1.50	2.5	125

*Based on a linear range of 0.5–2.0 mg ml^{-1} for each oestrogen.
†Calculated at 10% peak height.
‡Limit of detection based on a signal to noise ratio of 3 and an injection of approximately 20 nl.

one in which they were esterified, and finally, a single entity dosage form containing only β-oestradiol. In the conjugated or esterified dosage forms, the oestrogens were converted into the 'free' oestrogens with acid hydrolysis, extracted into chloroform, and injected into the SFC. It was discovered that, based on a 20 nl injection and limited solubility of the oestrogens in chloroform (<1.5 mg ml^{-1}), only the oestrone levels could be quantified in the conjugated or esterified dosage forms. In each dosage form, the concentration of oestrone was calculated by single point analysis based on a standard oestrone solution. In a dosage form containing conjugated oestrogens and chlordiazepoxide (Menrium, Roche), the oestrone concentration was found to be 0.320 ± 0.003 mg (RSD 0.9%, n = 3) compared to a labeled amount of 0.400 mg total conjugated oestrogens. In the dosage form containing esterified oestrogens and methyltestosterone (Estratest, Solvay), the oestrone level was calculated to be 0.987 ± 0.025 mg (RSD, 2.5%, n = 3) compared to the labelled amount of 1.25 mg total esterified oestrogens. A peak for equilin was detected, but was below a quantifiable level (<500 μg ml^{-1}). For interest, the methyltestosterone concentration in the combination dosage form was also calculated. The drug was extracted from the tablet with methylene chloride and then assayed by the SFC method using single point analysis based on a methyltestosterone standard solution. The amount of methyltestosterone found was 2.43 ± 0.025 mg (RSD, 1.03%, n = 3) compared to the labelled amount of 2.5 mg. The final oestrogen-containing dosage form to be assayed was a tablet labelled to contain 2.0 mg of β-oestradiol (Estrace, Mead Johnson) (see Fig. 3). The β-oestradiol level was found to be 1.99 ± 0.036 mg (RSD, 1.8%, n = 3). A comparison of the SFC data to an HPLC assay for β-oestradiol is shown in Table 4. There is no statistical difference in the SFC vs HPLC assay results for oestradiol in the dosage form at the 95% confidence level.

An SFC chromatogram shown in Fig. 4 indicates that a mixture of methyltestosterone and the five oestrogens can be separated on the cyanopropyl column using the multilinear density program developed for the oestrogens. When we attempted to use the method to analyse for methyltestosterone and oestrone concurrently in the Estratest dosage form, no SFC response for methyltestosterone was obtained even though the methyltestosterone level was well above the SFC detection limits of 125–150 μg ml^{-1}. It was found that the acid hydrolysis conditions used for the esterified oestrogens also degraded the methyltestoster-

Table 4
Comparison of oestradiol tablet* analysis by SFC and HPLC assays

Labelled amount	Amount found, SFC	Amount found, HPLC†
2.00 mg	1.99 ± 0.036‡ mg (RSD 1.8%)	2.07 ± 0.029 mg (RSD 1.4%)

*Estrace, Mead Johnson, Evansville, IN Lot MJC42.
†See ref. 23.
‡Mean ± standard deviation based on n = 3.

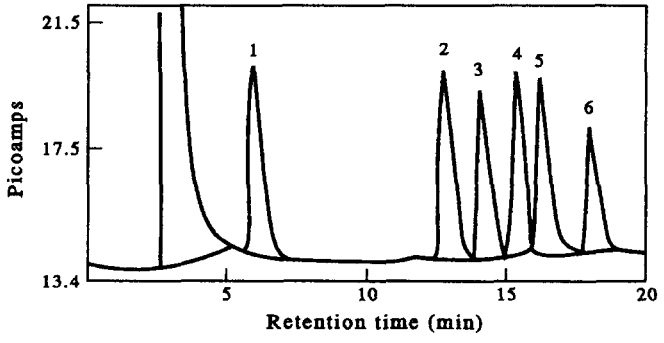

Figure 4
Typical SFC separation of methyltestosterone (1), oestrone (2), equilin (3), α-oestradiol (4), β-oestradiol (5), and *d*-equilenin (6) on a SB-cyanopropyl-50 column.

one and thus made it impossible to detect and quantify the intact drug concurrent with the oestrogens.

Conclusions

The results presented herein show that SFC is amenable to the chromatographic separation of oestrogens on the cyanopropyl capillary column using a carbon dioxide mobile phase and FID detection. The selectivity of the separation is very dependent on the carbon dioxide density and oven temperature. The method could be adapted to other pharmaceutical formulations containing conjugated, esterified, or 'free' oestrogens. Future improvements in SFC injection hardware will allow the detection and quantitation of the trace oestrogens that could not be determined in this study.

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