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Research Article

Estradiol Solubility in Aqueous Systems: Effect of Ionic Concentrations, pH, and Organic Solvents

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This study examined the effects of ionic strengths of NaCl (0.1, 0.3, and 1.0 M), pH (3, 7, and 11), and organic solvents (dichloromethane, diethyl ether, and methanol) on the extraction of estradiol at concentrations of 5.0 pg/mL in human serum. Methanol extracted almost 100% of the estradiol at a 5.0 pg/mL concentration, while ether and dichloromethane extracted only 73% or 70%, respectively, of the estradiol. The methanol extracted material was subjected to reverse phase high-performance liquid chromatography (HPLC) using 60% methanol and was found to elute at the same position as estradiol standard. These results suggest that methanol extraction of estradiol may prove useful in situations where estradiol occurs at concentration levels of ≥5.0 pg/mL, concentrations of great clinical significance in the detection and treatment of breast cancer.

1. Introduction

The routine measurement of serum estradiol levels in postmenopausal women requires an assay with a sensitivity of less than 5.0 pg/mL because postmenopausal women produce estradiol but not from their ovaries and therefore have extremely low serum levels [1]. There are practical analytical limitations to the estradiol assay when applied to clinical settings where levels may be ≤5.0 pg/mL. Present methods of estradiol analysis offer sensitivity of >20.0 pg/mL, which clearly points to the need for better routine methods of estradiol extraction and analysis at concentrations of 5.0 pg/mL or less [2]. The present study examined the effect of ionic strengths of NaCl (0.1 M, 0.3 M, and 1.0 M), pH (3, 7, and 11), and organic solvents (dichloromethane, diethyl ether, and methanol) on the extraction and analysis of estradiol at concentrations of 5.0 pg/mL.

2. Materials and Methods

2.1. Materials. All chemical reagents and solvents were obtained from Fisher Scientific Co. (Valley Stream Parkway, Malvern, PA, USA). ^{125}I labeled estradiol was obtained from Diagnostic Systems Laboratories, Inc. (Webster, TX, USA).

2.2. Methods

2.2.1. Preparation of Stripped Serum. The whole and "stripped" human serum was prepared at Massachusetts General Hospital, Boston, MA, USA. Briefly, 100 mL samples of freshly prepared serum were mixed with 30 grams of activated charcoal (G-60) and 6.0 grams of dextran (Pharmacia) for 30 minutes at 4°C. The mixture was centrifuged at 20,000 ×g for 30 minutes at 4°C.

The supernatant was passed through a 0.45 micron filter and stored frozen until use. The concentration of estradiol in this original stock prior to dilution was determined as described elsewhere [3, 4].

2.2.2. Extraction Procedures. Diethyl ether, dichloromethane, and methanol were used for extraction of estradiol standards in spiked control serum sample. Three concentrations of NaCl (0.1, 0.3, and 1.0 M) were also added to the mixtures. The extractions were also performed at three different pHs

(3.0, 7.0, and 11.0). Each extraction was performed six times, and the data was analyzed using Microsoft Excel 2.0 during the test function conditions.

Five mLs of each organic solvent were added to 1.0 mL serum samples that contained ^{125}I labeled estradiol (1 microcurie) and estradiol (5 pg/mL). The samples were vortexed and then allowed to separate (ether and dichloromethane) or subjected to centrifugation, 3000 ×g for 15 minutes at 23°C (methanol). The organic layers were removed by pipetting, and the resulting pellets were extracted two more times using the same volume of organic solvent. The extracts (aqueous layers) were combined, dried under nitrogen gas, and the residues were dissolved in methanol. Radioactivity in both the pellets and the extracts was measured using a Wizard gamma counter (Perkin Elmer, Boston, MA, USA).

In order to determine if the recovered radioactivity remained attached to estradiol, the methanol extracts were dried under nitrogen, resuspended in 10% (volume/volume) methanol, and analyzed by HPLC (Waters 510/Waters 486, Waters Inc., Milford, MA, USA) using a reverse phase C_{18} column (Waters W10951Q046 Symmetry C_{18} 5 micron Waters Inc., Milford, MA, USA). The column was eluted with 60% methanol. One mL fractions were collected using a fraction collector (FRAC-100, Pharmacia Inc., New York, NY, USA), and radioactivity was measured as previously mentioned.

2.2.3. Statistical Methods. Statistical analysis was performed by analysis of variance (ANOVA) followed by comparison of individual means using Duncan's new multiple range test. P values of <0.05 were considered to be significant.

3. Results

Figures 1 and 2 illustrate the extraction efficiency (performance) for the analytical removal of the ^{125}I spiked estradiol tracer from serum samples. The values plotted are the mean values for the combined data from these extractions with the standard deviation bars. Ether resulted in recoveries averaging 73% (Figure 1). Dichloromethane resulted in recoveries averaging 70% (Figure 1). As pH and salt concentration increased, the recovery of radioactivity in the pellet fraction increased, and the amount recovered in the extract decreased (Figure 2). However, the effect was not as prominent as with ether. Methanol produced almost 100% recovery of the added estradiol (Figure 1). The differences between the extraction by methanol compared to ether or dichloromethane, regardless of NaCl concentration and/or pH, were statistically significant ($P < 0.01$). The most statistically significant ($P < 0.01$) recovery as calculated using the ANOVA program was obtained with methanol combined with 0.1 M NaCl and pH 7.0. Two-way ANOVA demonstrated highly significant main effects of treatment, $F3, 160 = 48.06$ $P < 0.00001$ and pH, $F7, 160 = 9.90$; $P < 0.00001$.

As shown in Figure 2, with all the three organic extraction solvents, residual estradiol was found in the pellet fraction. Combining the percentage recovery of the extracts with the radioactivity found in the pellets demonstrated approximately 100% recovery for the methanol system but not for the ether or dichloromethane.

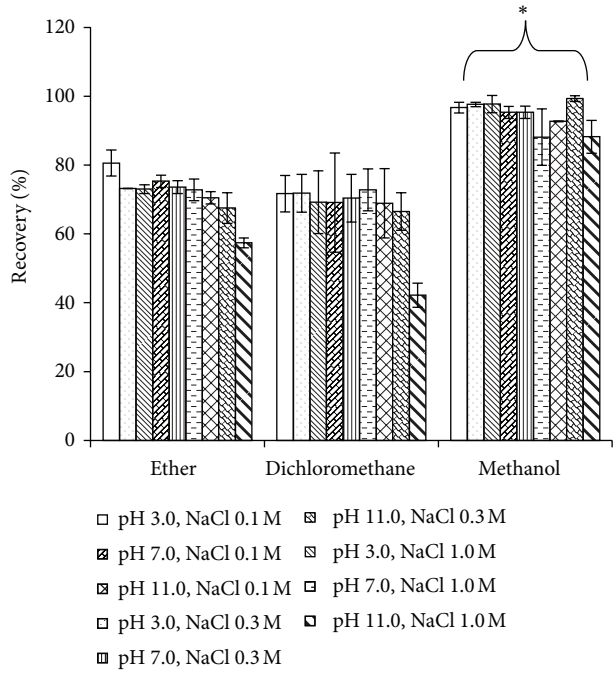

Figure 1: Percentage recovery of radiolabeled estradiol in the extracted fraction (mean ± standard deviation). Replicate serum samples containing ^{125}I labeled estradiol (1 microcurie) and estradiol (5 pg/mL) were subjected to extraction by each of the three solvents under the various pH and sodium chloride conditions as described in methods. The samples were subsequently spun, and the percentage of radioactivity in the supernatants was determined. The percentage of radioactivity recovered is shown here. The data represents the results of six separate experiments.

The ^{125}I estradiol that was extracted from 1 mL of the tracer using methanol was dried down, resuspended in 10% methanol, and injected into the HPLC as described in the previous experimental procedure. A single peak was recovered between tubes 57 and 62 which contained approximately 99% of the radioactivity added. When the methanol extracts from the spiked serums were run on the same column, they showed the same peak of activity in tubes 57 to 62 with essentially 99% recovery of the radioactivity added. These data suggest that the radioactivity that was recovered by the methanol extraction of serum remained associated with estradiol.

4. Discussion

Estradiol [5, 6] (C_{18} H_{24} O_2, also identified as estra-1,3,5 (10)-triene-3,17β-diol,17β-estradiol, or E_2) is the most potent hormone secreted by the ovaries during normal premenopause in women [7]. Estradiol was the last major natural hormone isolated from tissues in 1935 when only 12.0 mg was extracted from four tons of porcine ovaries [5]. Estradiol is responsible for the reproductive epithelia, breast development, maturation of long bones, and the development of secondary female sex characteristics. Estradiol may also serve as a protective and trophic factor for the brain [8]. Estradiol levels in plasma have been used for assessing fertility, amenorrhea, and precocious puberty in young females [4]. After menopause, when

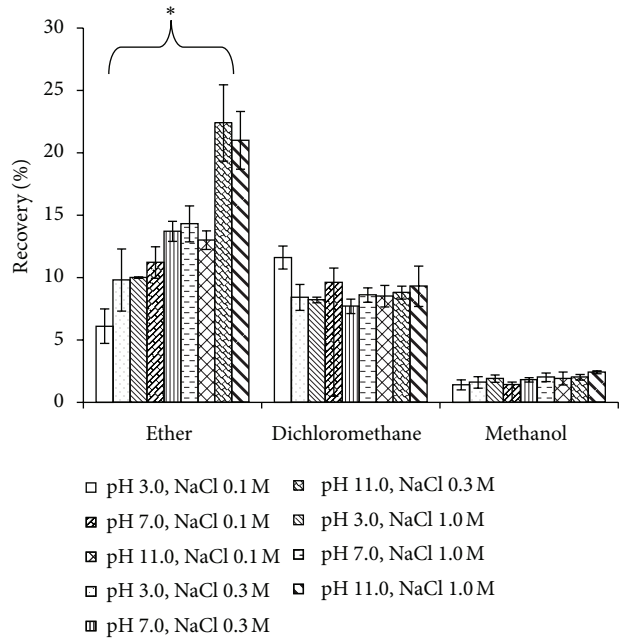

Figure 2: Percentage recovery of radiolabeled estradiol in the pellet fraction (mean ± standard deviation). The radioactivity in the pellet fractions of the samples described in Figure 1 was counted. The percentage of radioactivity recovered is shown here. The data represents the results of six separate experiments.

women's ovaries cease estrogen production, estradiol may be administered to replace the hormone [5]. Accordingly, estradiol supplementation may be given to prevent side effects of menopause.

There have been recent reports that estradiol may influence the development of cancer [7]. Estradiol increases cell proliferation and delays cell death, both of which may increase cancer risks. It has been suggested that the measurement of serum estradiol in postmenopausal women might help identify those women at high risk of breast cancer [7]. In fact, Cummings [7] found that women with high estradiol levels were 6.8 times more likely to develop breast cancer over four years compared with women with normal estradiol levels.

Estradiol implants have also been administered to hormone-treated cattle, leading to increased estradiol concentrations in meat obtained from these animals when compared to untreated cattle [9]. Estradiol has also been found in the sludge of waste treatment plants [10] which provides an opportunity for it to reenter potable water supplies or the active food chain. These xenobiotic sources might increase exposure to estradiol and raise serum hormone levels in the general population. Therefore, accurate measurement of estradiol concentrations in serum has great significance in veterinary as well as human medicine.

A number of methods have been described for extracting estradiol from blood since its initial isolation from urine in 1929 [11]. Many methods involve an extraction step followed by separation on a gas chromatograph (GC) followed by a mass sensitive detector (MS) [9], [12–20]. Such methods of analysis require the preparation of a volatile estradiol derivative. Immunoassay techniques can also be used for analysis of serum estradiol levels [3, 21–23]. In these procedures, an antibody to estradiol must be prepared. The estradiol molecule must have a radioisotope chemically attached to it that allows for analysis of estradiol in serum samples. Vega-Morales et al. [24] have recently described a very sensitive assay for estradiol which requires 250 mL of sample volume which is not applicable to routine clinical analysis of patient samples.

The Siekmann method [12] employs dichloromethane to extract the estradiol from serum, while the method of Wu et al. [13] uses diethyl ether. One disadvantage of the Siekmann method is that the dichloromethane, which is heavier than water, must be withdrawn from the bottom of an extraction tube. This must be done by puncturing the bottom of the extraction tube and letting the dichloromethane layer drip out or by carefully placing a pipette down the side of the tube through the water layer and into the dichloromethane layer. On the other hand, the Wu method [13] has a disadvantage in that it uses diethyl ether for the estradiol extraction. Diethyl ether is difficult to handle at room temperature, in part, because it has a low boiling point, and the extraction must be carried out in a fume hood.

In the present study, we used the salting out principle, as well as organic solvents, to extract estradiol at a clinically relevant low concentration (5.0 pg/mL). Salting out is a method of separation of proteins or hormones based on the principle that these compounds are less soluble at high salt concentrations and pH. Proteins contain hydrophobic and hydrophilic amino acids. After proteins unfold in aqueous solutions, the hydrophilic amino acids interact with the molecules of solvation allowing the proteins to form hydrogen bonds with the surrounding water molecules. When enough of the protein surface is hydrophilic, the protein is dissolved in water. When the salt concentration is increased and/or the pH is changed, the water molecules interact with the salt ions, decreasing the water interacting with the protein, resulting in protein-protein interactions that are stronger than the solvent-solute interactions, so that the protein molecules coagulate and the protein drops out of solution.

The present study examined the effects of ionic strengths of NaCl (0.1, 0.3, and 1.0 M), pH (3, 7, and 11), and organic solvents (dichloromethane, diethyl ether, and methanol) on estradiol extraction at concentrations of 5.0 pg/mL in human serum. Methanol extracted almost 100% of the estradiol at a 5.0 pg/mL concentration, while ether or dichloromethane extracted only 73 or 70% respectively, of the hormone. The methanol extracted material was subjected to reverse phase high-performance liquid chromatography (HPLC) using 60% methanol and was found to elute at the same position as estradiol standard. The results of this research suggest that methanol extraction of estradiol may prove useful in situations where estradiol occurs at concentration levels of 5.0 pg/mL which is a concentration of considerable clinical significance for the detection and treatment of breast cancer in postmenopausal women [7].

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