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CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC EVALUATION OF PROGESTERONE AND ESTROGEN

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Abstract

Steroid hormones viz. progesterone, estrogen were estimated through TLC in a concentration and time dependent manner i.e. 2.5mg/ml, 5.0mg/ml for 30 & 45 minutes and, 0.2mg/ml, 0.4mg/ml for 30 & 45 minutes respectively. Progesterone and estrogen were stained with 50% v/v aq. solution of Conc. H2SO4 and were estimated through TLC in a concentration and time dependent manner. Different steroid hormones travel at different rates due to the differences in their attraction to the stationary phase and because of differences in solubility in the solvent. These Rf values obtained from both the hormones were then compared and it was found that there was a reasonable difference. Further, a study on the interaction of steroid hormones with fatty acids and proteins was undertaken using a spectrophotometer. Steroid hormones viz. progesterone and estrogen were made to interact with measured amounts of alcohol, stearic acid and bovine serum albumin (BSA) and their absorbance were recorded at the excitation wavelength of 410 nm using a spectrophotometer. Progesterone (conc.2mg/ml) and estrogen (conc. 0.5mg/ml) were each mixed with 0.1 ml, 0.2ml and 0.4ml of stearic acid (conc. 0.5mg/ml) and 5 mg, 10 mg and 15 mg of BSA separately and their absorbance were noted at 410nm. A slight shift in the absorbance was found on the overall interaction of steroids: progesterone and estrogen with alcohol, stearic acid and BSA respectively, when excited to 410 nm. Thus an attempt was made to establish a valid spectrophotometric procedure for the study of interaction of steroid hormones with fatty acids and proteins.

Keywords: Progesterone, Estrogen, Stearic acid, Bovine serum albumin, Chromatography

Introduction

Steroids are a class of compounds that have a cyclopentano-perhydro-phenanthrene skeleton and that occur in nature and in synthetic products. The bile acids, androgens, estrogens, corticosteroids, ecdysteroids, sterols and vitamin D are compounds included in the class of steroids. Steroids and their metabolites are analyzed by thin-layer chromatography (TLC) in a variety of samples such as biological samples or plants and pharmaceutical formulations. TLC continues to be an important method for the determination of steroids because of its advantages. Many samples can be analyzed simultaneously and quickly at relatively low cost, multiple separation techniques and detection procedures can be applied and the detection limits are often in the low nanogram range and quantitative densitometric methods are accurate. The albumin-mediated hepatic uptakes of free fatty acids [1], bile acids and Rose Bengal [2] have been reported, but the kinetics are not sufficiently clear. It was suggested by some experimental results *in vitro* using a liquid membrane system (hexane source phase/bovine serum/hexane receiving phase) that the rate of uptake of steroids from blood to intracellular space is controlled not only in the free form but also in forms bound with serum proteins such as albumin [3]. Nowadays, it is commonly accepted that the fractions of steroids which bind with high affinity to plasma proteins such as globulins are less easily available to tissues. Although steroids bind with low affinity to human serum albumin (HSA), most of the circulating steroids were bound to HSA due to the high concentration of albumin [4]. Recent studies have shown that, in addition to free steroids, those bound to albumin in plasma may also be available to tissues [5]. The binding of steroids to albumin is affected by temperature, pH, fatty acid and competing ligands. Plasma levels of FFA vary throughout the day [6] and the availability of steroids to tissues is changed by FFA binding to albumin. Serum albumin is the most abundant protein in the circulatory system, accounting for 60% of the total serum protein . In mammals, albumin is synthesized by the liver and possesses a half-life in circulation of 19 days. Its principal function is to transport fatty acids, a great variety of metabolites and drugs such as anti-coagulants, tranquilizers and general anesthetics. Serum albumin has been one of the most studied proteins for over 40 years because its primary structure is very well known for a long time and

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its tertiary structure was determined a few years ago by X-ray crystallography.

It is known that progesterone and estrogen were bound to the same site on serum albumin (SA) [7] and consequently a competition effect exists probably between these two steroid sex-hormones. Then, even if *in vivo*, the balance between the progesterone and the estrogen rate was controlled by multiple mechanisms and Gleason's observation could be explained by an enhancement of the progesterone displacement to its SA binding site by estrogen when Mg^{2+} cation concentration increased [8]. SA is the major plasma protein responsible for the reversible binding of a wide range of drugs [9]. Extensive studies on different aspects of drug–SA interactions are still in progress because of the clinical significance of the process, especially in the case of tightly bound drugs (K > 105M−1) [10]. Numerous analytical techniques are used for protein binding studies and they are continuously being added to, along with extending knowledge about the complex mechanisms involved in the drug–SA binding process [11]. It has been recognized that an inverse relationship exists between the number of polar groups in a steroid and the strength of interaction with serum albumin. With the aid of a spectrophotometric procedure (12), the validity of which was established by a comparison with the method of equilibrium dialysis, the influence of structural alterations in sex steroids on the interaction with bovine serum albumin (BSA) was investigated. It was found that introduction of 0x0 or hydroxyl groups in various positions weakens this interaction, and that introduction of methyl groups has the opposite effect. Characteristic differences were noted for CY substituents in their influence on the strength of association. Furthermore, the elution profiles of gel-filtration chromatography clearly showed that progesterone and testosterone are easily liberated from the steroid/BSA complexes and that FFA potentiates the binding of these steroids to BSA. In the case of HSA, the binding affinities of progesterone and testosterone were not greatly affected by bound FFA [13,14].

This study was undertaken towards chromatographic evaluation of progesterone and estrogen using the H_2SO_4 staining method and spectrophotometric analysis of the interaction of both the sex steroids with BSA and stearic acid.

Materials and Methods

Drugs and chemicals

Progesterone, Estrogen, Silica gel (ACME Chemicals, India), Sulphuric acid reagent, benzene, ethyl acetate, bovine serum albumin, stearic acid, ethanol, propylene glycol

TLC equipment

All the equipments used for TLC used were from ACME synthetics, Mumbai, India. Acme applicator, TLC glass plates, Beakers, Capillary pipettes for spotting solutions, Rulers, hot plates, Gloves were used for all chemical usage.

Thin layer chromatography

This is a chromatography technique used to separate mixtures. Thin layer chromatography is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose. This layer of adsorbent is known as the stationary phase.

After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up by the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved. In steroid analysis, TLC is the method of choice, especially when many simultaneous analyses have to be carried out, hundreds of analyses can be performed in a short time and with small demands on equipment and space. Samples can be analyzed with minimal clean up, and analyzing a sample by the use of multiple separation steps and static post-chromatographic detection procedure is also possible because all sample components are stored on the layer without the chance of loss. The time required in TLC analysis is about 10-60 min. As little as 0.001 µg of steroids/spot can be detected by TLC. Using a TLC plate with thicker adsorbent layers (0.5-2 min), several grams of substance can be isolated.

Sulphuric acid staining method

After plate development, the plate is dried and sprayed with a 50% v/v aqueous solution of sulfuric acid in a fume hood. The hood was exposed to extremely corrosive materials and, thus, all fittings were made of glass or acid resistant plastic. After spraying, and while still in the fume hood, the plate is heated to about 80˚C for about 45 minutes on a hot plate or in an oven. The solutes are partially oxidized leaving behind a charred deposit of black carbon that is easy to distinguish. This method will detect most *in volatile* organic compounds. Sulfuric acid spray reagent was found to be particularly suitable for differentiating natural estrogens from diethylstilbestrol in both a mixture of natural steroids and in biologic preparations under daylight as well as ultraviolet light.

Solvent system: Benzene and Ethyl acetate in the ratio of 5:1 was used.

Spectrophotometric evaluation

Spectrophotometric techniques are used to measure the concentration of solutes in solution by

measuring the amount of light that is absorbed by the solution in a cuvette placed in the spectrophotometer. Spectrophotometry takes advantage of the dual nature of light. Namely, light has:

1. A particle nature which gives rise to the photoelectric effect
2. A wave nature which gives rise to the visible spectrum of light.

The spectrophotometer can measure the amount of light (of certain frequency) transmitted or adsorbed by the solution. This light that has not been absorbed by the solution in the cuvette, will strike the phototube. The photons of light that strike the phototube will be converted into electrical energy. This current that is produced is very small and must be amplified before it can be efficiently detected. The signal is proportional to the amount of light which originally struck the phototube and is thus an accurate measurement of the amount of light which has passed through (been transmitted by) the sample. Different compounds having dissimilar atomic and molecular interactions have characteristic absorption phenomena and absorption spectra. Concentration of every component may be found from the spectrophotometer measurements and calibration curve made using the samples of known concentration. In this study the spectrophotometer used was Ultrospec III (Pharmacia). All samples were analysed at an absorbance of 410 nm and optical density was recorded.

Sulfuric acid spray cabinet

Experimental protocols

1) Interaction of steroids with bovine serum albumin (BSA)

The sample solution consists of progesterone and estrogen mixed each with stearic acid and BSA in three different concentrations and the absorbance is noted at 410 nm.

a. 2ml progesterone (2mg/ml) + 1 ml stearic acid (0.5mg/ml) + 5mg BSA
b. 2ml progesterone (2mg/ml) + 1 ml stearic acid (0.5mg/ml) + 10mg BSA
c. 2ml progesterone (2mg/ml) + 1 ml stearic acid (0.5mg/ml) + 15mg BSA
d. 2ml estrogen (0.5mg/ml) + 1 ml stearic acid (0.5mg/ml) + 5mg BSA
e. 2ml estrogen (0.5mg/ml) + 1 ml stearic acid (0.5mg/ml) + 10mg BSA
f. 2ml estrogen (0.5mg/ml) + 1 ml stearic acid (0.5mg/ml) + 15mg BSA

2) Interaction of steroids with alcohols

The sample solution consists of progesterone and estrogen mixed each with ethanol and propylene glycol in the following concentrations and the absorbance of each sample is noted at 410nm.

a. 0.5ml progesterone (2mg/ml) + 2 ml ethanol
b. 0.5ml progesterone (2mg/ml) + 2 ml propylene glycol
c. 0.5ml estrogen (0.5mg/ml) + 2 ml ethanol
d. 0.5ml estrogen (0.5mg/ml) + 2 ml propylene glycol

3) Interaction of steroids with stearic acid

The sample solution consists of progesterone and estrogen mixed each with ethanol and stearic acid in three different concentrations and the absorbance is noted at 410 nm.

a. 0.5ml progesterone (2mg/ml) + 2 ml ethanol + 0.1 ml stearic acid(0.5mg/ml)
b. 0.5ml progesterone (2mg/ml) + 2 ml ethanol + 0.2 ml stearic acid(0.5mg/ml)
c. 0.5ml progesterone (2mg/ml) + 2 ml ethanol + 0.4 ml stearic acid(0.5mg/ml)
d. 0.5ml estrogen (0.5mg/ml) + 2 ml ethanol + 0.1 ml stearic acid(0.5mg/ml)
e. 0.5ml estrogen (0.5mg/ml) + 2 ml ethanol + 0.2 ml stearic acid(0.5mg/ml)
f. 0.5ml estrogen (0.5mg/ml) + 2 ml ethanol + 0.4 ml stearic acid(0.5mg/ml)

Results

The results of experiments conducted with chromatographic and spectrophotometric evaluation of progesterone and estrogen are depicted in Table 1 & 2 and Figures 1 & 2. The results show appreciable difference in Rf values of progesterone and estrogen depending on the elution time. Similarly the addition of BSA to the reaction mixture of progesterone and estrogen showed appreciable difference in optical density as depicted in Figures 1 & 2. Stearic acid (0.5mg) was also able to shift the absorbance values of progesterone.

Table 1: Table 1 describes the thin layer chromatographic evaluation of progesterone (2.5 & 5 mg) conducted using the concentrated H_2SO_4 staining technique and eluted for a duration of 30 and 45 minutes. The last column of the table shows the Rf values obtained.

S.no	Progesterone conc. (mg/ml)	Time duration (min)	Dist. moved by solute (cm)	Dist. moved by solvent (cm)	Retention factor (Rf)
1	2.5	30	6.6	7.2	0.916
2	2.5	45	9.3	9.8	0.948
3	5.0	30	6.7	7.2	0.930
4	5.0	45	9.5	9.8	0.969

a. 0.1ml progesterone (25mg/ml) + 50% v/v aq. solution of Conc. H_2SO_4
b. 0.2ml progesterone (25mg/ml) + 50% v/v aq. solution of Conc. H_2SO_4
SOLVENT SYSTEM: Benzene and Ethyl acetate in the ratio of 5:1

Table 2: Table 2 describes the thin layer chromatographic evaluation of estrogen (0.2 & 0.4 mg) conducted using the concentrated H2SO4 staining technique and eluted for a duration of 30 and 45 minutes. The last column of the table shows the Rf values obtained.

S.no	Estrogen conc. (mg/ml)	Time duration (min)	Dist. moved by solute (cm)	Dist. moved by solvent (cm)	Retention factor (Rf)
1	0.2	30	6.6	7.8	0.858
2	0.2	45	6.7	8.2	0.804
3	0.4	30	6.8	7.8	0871
4	0.4	45	6.8	8.2	0.829

a. 0.2ml estrogen (1mg/ml) + 50% v/v aq. solution of Conc. H_2SO_4
b. 0.4ml estrogen (1mg/ml) + 50% v/v aq. solution of Conc. H_2SO_4
SOLVENT SYSTEM: Benzene and Ethyl acetate in the ratio of 5:1

Figure 1: Figure 2 describes the optical density values at absorbance of 410 nm obtained with estrogen (0.25 & 1 mg), ethanol (2ml), bovine serum albumin (10mg) and stearic acid (0.5 mg) alone or in combination.

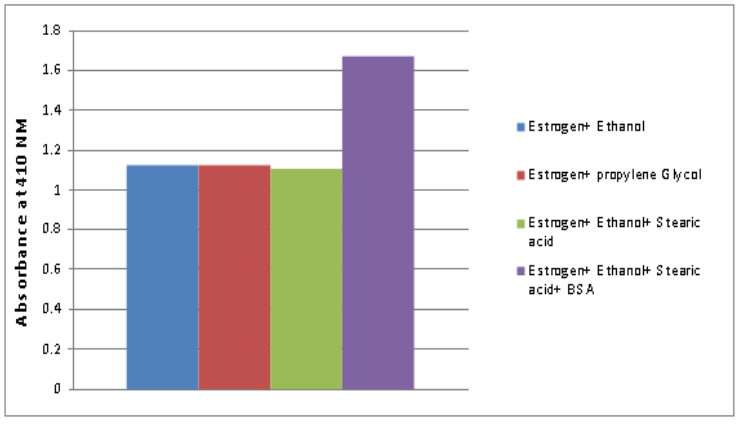
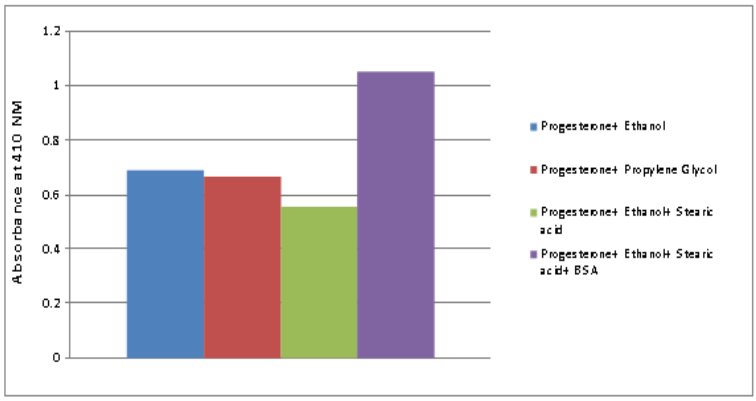


Figure 2: This figure describes the optical density values at absorbance of 410 nm obtained with progesterone (1 & 4 mg), ethanol (2ml), bovine serum albumin (10mg) and stearic acid (0.5 mg) alone or in combination.



Discussion

Steroids and their metabolites are analyzed by thin-layer chromatography (TLC) in a variety of samples such as biological samples or plants and pharmaceutical formulations. TLC continues to be an important method for the determination of steroids because of its advantages [15].Many samples can be analyzed simultaneously and quickly at relatively low cost, multiple separation techniques and detection procedures can be applied and the detection limits are often in the low nanogram range and quantitative densitometric methods are accurate. The results of this study suggest the novel mechanism using concentrated H_2SO_4 to identify the eluted progesterone and estrogen on a thin layer chromatography plate. The results of the TLC obtained were satisfactory and although this technique is comparatively old but can be applied to find out the Rf values of the steroids in a simple manner. The benzene and ethyl acetate combination proved to be an effective mobile phase. There was some difference in the Rf values based on the time duration of TLC but it was marginal. The results are shown in Table 1 and Table 2.

To evaluate the effect of fatty acid and protein, stearic acid and BSA, we conducted studies on the absorbance using the spectrophotometer. Our results suggest that addition of BSA to the reaction mixture caused significant change in optical density in both steroids i.e progesterone and estrogen (Fig.1 & 2). The cellular effects elicited by estrogen and progesterone [16] covalently conjugated to membrane impermeable BSA have been attributed to non-genomic actions mediated by membrane associated hormone receptors. It has previously been established that for physiological concentrations of progesterone and albumin in the circulating blood, approximately 99% of the steroid is bound to albumin. If more than one molecule of the protein participated in the binding of one molecule of progesterone, dimerization or complexing of more than two albumin molecules would result and should be demonstrable by the viscosity and by the sedimentation behavior of the system. Determination of viscosity and sedimentation constants under the conditions outlined in the experimental part failed to reveal any differences between the albumin solutions with and without progesterone. It was concluded from these findings that only one molecule of human serum albumin complexes with one molecule of progesterone. It would seem reasonable to assume that a similar molar relationship exists in the interaction of other steroid molecules with serum albumin. The steric relationship between the two components in the complex was investigated by measuring the strength of interaction between albumin and epimeric steroids. Such a comparison should permit conclusions as to the spatial arrangement of the interacting molecules. Thus there are multiple reasons for the change in absorbance with addition of BSA to the reaction mixture [17].

Furthermore, fatty acid tends to minimize or eliminate the well-known differences in affinity between bovine and human albumin for interactions with these two steroids [18]. The values for binding affinity in the interaction of testosterone with these batches of human serum albumin are significantly higher than those previously published by some authors and the value for progesterone-bovine albumin interaction is not in accordance with the "polarity rule". Studies of these same interactions by ultraviolet difference spectroscopy give further evidence of the augmentation in binding but, in the case of defatted bovine albumin only, the aromatic difference troughs are indicative of tyrosine perturbation whereas refatted bovine albumin, defatted and refatted human albumin manifest tryptophan perturbation. Quantitative correlation of perturbation with level of bound steroid suggests that fatty acid alters the ratio (possibly hydrogen-bonded to non hydrogen-bonded) of two forms of bound steroid. This concept gains in validity

when one considers the nature of the binding forces between steroid and protein. For lack of specific knowledge as to these forces, the binding between neutral steroids and proteins may best be interpreted as mediated by hydrogen bonds and van der Waals forces [19]. Common to both of these binding mechanisms is the low energy of the bond, of the order of 5 to 10 kcal per mole, which explains the ready dissociation of the complexes. Furthermore, they are highly dependent upon the distance; the van der Waals forces for spherical atoms are inversely proportional to the seventh power of the distance between the atomic centers. For efficient interaction, therefore, a close fit of the steroid and protein surfaces would be required.

Whether the presence of stearic acid alone in any way alters chemical configuration and affects the absorbance by spectrophotometer can only be ascertained by specific chemical tests. The change in stearic acid mediated absorbance with progesterone can be attributed to interplay of chemical forces between the steroid and the fatty acid. Studies with lauric, myristic, and palmitic acid showed that the decrease of binding affinity for progesterone was proportional to the amount of fatty acid added to albumin, and to its chain length. These results confirm and extend other findings of inhibition of progesterone binding to human albumin by saturated fatty acids.

In conclusion this study describes a novel H_2SO_4 staining technique to visualize the sex steroids, progesterone and estrogen and a change in spectrophotometric absorbance of progesterone and estrogen by BSA and stearic acid.

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