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A COMPARATIVE ASSESSMENT OF THE EFFICIENCY OF HPLC COLUMNS AND CHROMATOGRAPHIC CONDITIONS IN LC-MS ANALYSIS OF DERIVATISED ESTROGEN HORMONES AND THEIR METABOLITES

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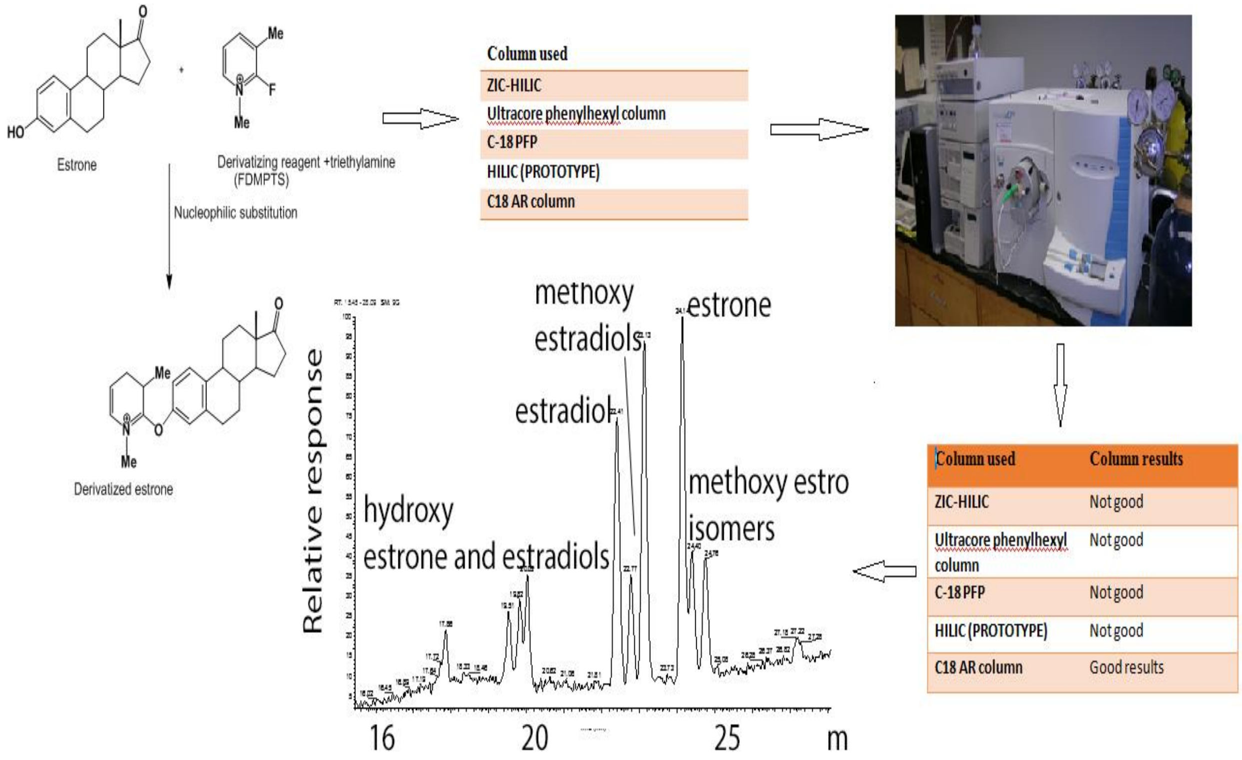
Article Info

Abstract

Determination of estrone, estradiol, and their metabolites is a very challenging task as its ionization and determination offer considerable difficulties. Different types of HPLC columns were used in different conditions in order to get the best separation. Derivatives of estrone, estradiol, and their metabolites were produced by using a derivatization agent 2-Fluoro-1, 3-dimethylpyridinium p-toluene sulfonate, followed by the chemical analysis using different types of columns. Different combinations of mobile phases were also investigated and the best separation was achieved with Mobile phase A and B consisted of 0.1% v/v formic acid in water and acetonitrile respectively, used in a gradient method. Different concentrations of estrone, estradiol and their metabolites for calibration curves were prepared, derivatized with the reagent and were analyzed by LC-MS technique. The derivatives produced by this method have shown several times higher detection response. Using this derivatization method, spiked standards of estrone, estradiol and their metabolites were analyzed and the method was then applied to culture media samples for determination of estrogens hormones. The results suggested that the best resolution of estrogens and their metabolites and peak shapes were obtained by using C18-AR columns and Thermo Orbitrap mass spectrometer as compare to the other HPLC columns.

Keywords:
LTQ Orbitrap; Mass Spectrometer; derivatization; liquid chromatography; estrone; estradiol.



Column used
ZIC-HILIC
Ultracore phenylhexyl column
C-18 PFP
HILIC (PROTOTYPE)
C18 AR column

Column used	Column results
ZIC-HILIC	Not good
Ultracore phenylhexyl column	Not good
C-18 PFP	Not good
HILIC (PROTOTYPE)	Not good
C18 AR column	Good results

Introduction

Estrogen is a female sex hormones, having an important role in the development and maintenance of the female reproductive system and are responsible for controlling the secondary gender characteristics of females (1, 2). Along with other steroid hormones, estrogen possesses a very potent biological activity in the body even in very low concentration (i.e., in picograms), and these hormones (both natural and synthetic) are using for range of therapeutic purposes. Thus, a well-defined method is needed to determine the concentration of both endogenous and exogenous steroidal hormones in order to optimize the diagnosis and therapies of chronic disorders. Though, determining these hormones in the body is a challenging task because of their structural similarities and their low concentration in the body. Number of techniques has been used for the determination of these hormones across research studies. Liquid chromatography coupled with mass spectrometry (LC-MS) is the most recent technique employed for the determination of steroidal hormones because of its selectivity and versatility. However, the ionization capability of most of the steroids is not so good in mass spectrometry systems and is thus not satisfactory for the assessment of trace amounts of steroidal hormones (3-6)

Chemical derivatization has been found to be a powerful technique for enhancing the detection of the compound under investigation by making them more ionizable in the LC-MS system, via introducing a moiety into the structure resulting in the modification of the structure of target compounds changing their physical and chemical properties. The derivatization reagent is commonly selected on the basis of the functional group present in the structure of the target compound along with the research purpose. Following chemical derivatization, the physical and chemical characteristics of the target compound may alter affecting the ionization efficiency, retention time in liquid chromatography, stability and solubility of the compound. So it is actually the chemical structure of the target compound that may define the strategy for the selection of best the derivatization reagent (7).

There are many factors that can influence the production of ions and signals intensity in the mass spectrometry system (8). Electrospray ionization (ESI) has now become the most important technique for the production of gas-phase ions from a variety of compounds but the detection sensitivity of ESI is much poor for neutral steroids. The best detection of steroids can be obtained if the analyte is either in ionic form or can be readily ionized in solution (3). For this purpose, researchers have produced more ionizable derivatives by modifying the steroid structure in order to enhance the detection of non-ionizable compounds. As all estrogenic compounds have a phenolic group in the structure, this functional group can be employed to attach the atmospheric pressure ionization API sensitive moieties. In most of the derivatization techniques used for analysis in ESI, an ionizable group or permanently charged group may attach to the analyte to enhance the detection signals (3, 8). The derivatization of estrone estradiol with 2-Fluoro-1, 3-dimethylpyridinium *p*-toluene sulfonate is shown in **Figure 2 and 3**.

The current study is aimed to chemically analyse estrogen hormones and their metabolites via LC-MS analysis technique using different HPLC columns and chromatographic conditions, and to compare the effectiveness of HPLC columns in separation and determination of estrogen hormones.

Figure 1: Flow chart of derivatization method of estrogens and their metabolites

2. Materials and Methods

2.1 *Materials*

2-Fluoro-1, 3-dimethylpyridinium *p*-toluene sulfonate, Estradiol, estrone, estrone 13C2 and estradiol 2H5 were purchased from Sigma-Aldrich, UK. Estrogen metabolites- 1, 3, 5(10)-estratriene-3, 16α-diol-17-one (16alphaOHE1), 1, 3, 5(10)-estratrien-3, 16α, 17β-triol (16aplhaOHE2), 1, 3, 5(10)-estratrien-3, 16α, 17β-triol (16-epi-E3), 1, 3, 5(10)-estratrien-3, 17α-diol (17alphaE2), 1, 3, 5(10)-estratrien-3, 4-diol-17-one 4-methyl ether (4MeOHE1), 1, 3, 5(10)-estratrien-3, 4, 17β-triol 4-methyl ether (4MeOHE2), 1, 3, 5(10)-estratrien-2, 3-diol-17-one 2-methyl ether (2MeOHE1), 1, 3, 5(10)-estratrien-2, 3, 17β-triol 2-methyl ether (2MeOHE2), 1, 3, 5(10)-estratrien-3, 4, 17β-triol (4OHE2), 1, 3, 5(10)-estratrien-3, 4-diol-17-ONE (4OHE1) were provided by the University of Glasgow, UK. HPLC grade acetonitrile (ACN) was purchased from Fisher Scientific, UK. HPLC grade water was produced by a Direct-Q 3 Ultrapure Water System from Millipore, UK. AnalaR grade formic acid (98%) was obtained from BDH-Merck, UK. Methanol and triethylamine (TEA) were purchased from Sigma-Aldrich, UK. Strata C18-E (55 µm, 70 Å) cartridges were purchased from Phenomenex, Macclesfield, UK.

2.2 Preparation of Derivatization Reagent

A fresh solution of derivatization reagent, 2-Fluoro-1, 3-dimethylpyridinium *p*-toluene sulfonate, 5mg/ml was prepared in acetonitrile prior to each experiment. The preparation method of the derivatization reagent is shown in flow chart below in figure 1.

2.3 Preparation of standard solutions

Estradiol and estrone were dissolved in ACN to make a standard stock solution (1 mg/ml). Different concentrations were prepared from the standard stock solution for the experiment. In addition, internal standard IS solutions were prepared using ACN to make a 1 mg/ml solution.

2.4 Samples preparation and treatment

Media samples (cell culture medium from pulmonary artery cell cultures following incubation with estrogens) were prepared and provided by the University of Glasgow, UK. The samples were then kept at -20 ^{O}C until analysis.

2.5 Extraction of Estrogens from Media samples

The samples were extracted using solid-phase extraction. Different C18 columns were used in order to the best results for extraction. The results obtained with the Strata X polymer cartridges were very good. The Strata X cartridges were pre-conditioned with 1 ml of methanol and 1 ml of water. The samples were spiked with 10 ng of stable isotope-labeled internal standards before extraction. Then the samples (4 ml) were passed through the column followed by a washing step with water (1 ml). After washing with water, the cartridge was eluted with 2 ml of methanol. The eluent was collected and then evaporated to dryness under nitrogen. The samples were subsequently reconstituted in acetonitrile and run by LCMS system. Calibration curves were prepared by making different concentrations of steroids mixture and then derivatized with the reagent 2-fluoro-1, 3-Dimethylpyridinium p-toluene sulfonate.

2.6 Derivatization with 2-fluoro-1, 3-Dimethylpyridinium p-toluene sulfonate

The derivatization procedure as shown in Figure 1, was carried out by adapting the work of Mukai Yama et al. (9) with slight modifications. To the dried sample was added 50 µl of 1% TEA in Acetonitrile, followed by the addition of 50 µl freshly prepared derivatization reagent (5 mg/ ml in ACN). The mixture was vortexed for 10 seconds and then incubated for 15 minutes at 40 ^{O}C. After incubation 50 µl of mobile phase component A (0.1% Formic acid) was added to the mixture and vortexed. A volume of 10 µl was then injected into LC-MS for analysis.

2.7 Method validation

The method was validated according to ICH guidelines for linearity, accuracy and precision, reproducibility, sensitivity or LOD/LOQ and recovery.

2.8 LC-MS analysis

Measurement of standards and samples was carried out on an LTQ Orbitrap mass spectrometer from Thermo Fisher Scientific (Hemel Hempstead, UK). Different HPLC columns were tried for the best separation and detection. The column which gives the best result was an ACE 5 C18-AR column (150 × 4.6mm, 5µm Hichrom, Reading, UK). Mobile phase A consisted of 0.1% v/v formic acid in water and Mobile phase B consisted of 0.1% v/v formic acid in CAN, used in a gradient method. The gradient was increased from 5% to 95% for Mobile phase B in 30 minutes and then decrease back to its initial composition in 1 minute. The system was re-equilibrated for 5 minutes before the next injection. The flow rate was 300µl/min. The ESI interface was operated in a positive ion mode with a spray voltage of 4.5 kV. The temperature of the ion transfer capillary was 275 °C and the flow rates of the sheath and auxiliary gases were 50 and 17 arbitrary units, respectively. The full scan range was m/z 75 to 1200. The data was recorded using Xcalibur 2.1.0 software (Thermo Fisher Scientific). Mass calibration was analyzed using the standard Thermo Cal mix solution and the signal at 83.0604 m/z (2xACN+H) was selected as a lock masses for positive ion mode during each analytical run.

3. Results

The reaction of steroids with FDMPTS shown in **Figure 2 and 3** produced a permanently charged derivative through nucleophilic substitution which has a high detection capability in the ESI mass system due to the inherent positive charge (10) which may increase the detection sensitivity of the target analyte.

Figure 2: Derivatization of estrone with fluorodimethylpyridinium

Figure 3: Derivatization of estradiol with the fluorodimethylpyridinium

Different types of columns **(Table 1)** were tested for their efficiency in separating estrogen hormones, including Zic-Hilic column, ACE Ultra core 2.5 Superphenylhexyl (150 × 3 mm id), ACE 5 PFP Hilic (Prototype) (250 × 4.6 id) and ACE 5 C18-AR column (150 × 4.6mm, 5µm Hichrom, Reading, UK). Initially the samples were tested by using Zic-Hilic column on orbitrap mass spectrometer, but the separation on the Zic-Hilic column was poor and there were no peaks observed for the estrogens on the Zic-Hilic column. The results from Ultra core phenylhexyl column, C-18 PFP, and HILIC (PROTOTYPE) (250 × 4.6 id) columns were also not consistent and satisfactory, (chromatograms of these columns will be provided if requested) though some separation of estrone and estradiol on phenylhexyl column and C-18 PFP was observed.

Table 1: Summary of the columns and conditions used for determination of estrogens and their metabolites

Column used	**Conditions**	**Comments**
ZIC-HILIC	Mobile phase A 0.1% FA in water Mobile phase B 0.1% FA in acetonitrile Gradient used: 0 min 5% mobile phase B 30 min 95% mobile phase B 31 min 5% mobile phase B 36 min 5% mobile phase B Flow rate 0.3 ml/min Different composition of mobile phase A and B used isocratic ally	**The separation on the ZIC-HILIC column was poor and there was no peaks observed for the estrogens on ZIC-HILIC column**
Ultra core phenylhexyl column	Mobile phase A 0.1% FA in water Mobile phase B 0.1% FA in acetonitrile Gradient used: 0 min 5% mobile phase B 30 min 95% mobile phase B 31 min 5% mobile phase B 36 min 5% mobile phase B Flow rate 0.3 ml/min	**The results obtained from ultra core phenylhexyl column was not good and consistent, the separation was also poor.**

C-18 PFP	Mobile phase A 0.1% FA in water Mobile phase B 0.1% FA in acetonitrile Gradient used: 0 min 5% mobile phase B 30 min 95% mobile phase B 31 min 5% mobile phase B 36 min 5% mobile phase B Flow rate 0.3 ml/min	**The results was not satisfactory, no peaks were observed for the estrogen and their metabolites**
HILIC (PROTOTYPE)	Mobile phase A 0.1% FA in water Mobile phase B 0.1% FA in acetonitrile Gradient used: 0 min 5% mobile phase B 30 min 95% mobile phase B 31 min 5% mobile phase B 36 min 5% mobile phase B Flow rate 0.3 ml/min	**No any peaks even observed with HILIC (PROTOTYPE) column**
C18 AR **column**	**Mobile phase A 0.1% FA in water Mobile phase B 0.1% FA in acetonitrile Gradient used: 0 min 5% mobile phase B 30 min 95% mobile phase B** **31 min 5% mobile phase B** **36 min 5% mobile phase B Flow rate 0.3 ml/min**	**The results obtained was very good and satisfactory, both the samples and standard have the same retention times, the estrogen and their metabolites was detected in both media samples and standards solution and the results is also reproducible. So C 18 AR column was selected for this study**

The chromatograms below in **Figure 4** suggested that the best resolution of estrogens and their metabolites and peak shapes were obtained by using C18-AR columns and Thermo Orbitrap mass spectrometer. The estrone and estradiol and their metabolites were detected well in the samples and standards mixture, the separation pattern and peak shapes were also good. The method was then applied to the media samples (cell culture medium from pulmonary artery cell cultures following incubation with estrogens), resulting in the detection of the metabolites in some of the samples. This indicated that the derivatization method used in the study, C18-AR columns and Thermo Orbitrap mass spectrometer actually work for the accurate determination of estrogen hormones and their metabolites.

Figure 4: Separation of steroid mixture derivatized with DMP on a C18 AR column, the separation obtained with C18 AR column was very satisfactory, both estrogens and their metabolites were observed and match the standard solution of steroids mixture and confirmed by x-caliber software.

4. Discussion

The positive ion mass spectrum of the estrogens and their metabolites are shown in **Figures 5**. The chromatograms in **Figure 4** clearly indicated that they are well resolved by the C-18 AR column as compare to the other columns. There is an intense signal for estradiol and estrone obtained after derivatization at m/z 378 and m/z 376, respectively. The hydroxymethyl metabolites of estrone were detected at m/z 406 after derivatization. Hydroxy estrone metabolites are observed in some samples at m/z 392 after derivatization. Estradiol hydroxyl methyl metabolites were detected at m/z 408 in both the standard and samples. Estradiol hydroxyl metabolites were detected at m/z 394. Hence various metabolites of estradiol were also observed by this derivatization technique. Since the 1, 3-Dimethylpyridium derivative estradiol and estrone correspond in mass to $(M +106)^+$ where M is the mass of estrogens which is 272 and 270 in case of estradiol and estrone respectively. The labeled internal standard Estrone C13 and estradiol D5 was also detected in both standards and samples at m/z 379 and 383 respectively shown in **Fig 5**.

Figure 5: Mass spectrum of (**A**) DMP (fluorodimethylpyridinium) derivative of estradiol, (**B**)DMP (fluorodimethylpyridinium) derivative of estrone, (**C**) DMP (fluorodimethylpyridinium) derivative of hydroxy estradiol, (**D**) DMP (fluorodimethylpyridinium) derivative of methoxy estradiol, (**E**) DMP (fluorodimethylpyridinium) derivative of $^{13}C3$- estrone, (**F**) DMP (fluorodimethylpyridinium) derivative of $^{2}H5$- estradiol

The method was validated according to ICH guidelines. Initially, the method was applied by using standards spiked into the water in order to determine the limits of detection from the steroids. First of all, estradiol and estrone were used to test the method. Linearity of the method was determined by preparing different concentrations of estrone and estradiol and their metabolites ranging from 0.1 ng/ml to 6.4 ng/ml were prepared in a mixture and derivatized with the reagent according to the method mentioned above and were analyzed by LC-MS. The precision and accuracy of the method was done by selecting three concentrations and run them in triplicate, the chromatograms and detection sensitivity of higher and intermediate and low concentration was very good using this column and ranged from +/-1.2% to +/-3.9%. To determine reproducibility of the method, a single-point standards of 0.8 ng/ml and 1.6 ng/ml was run every day, and results were always well within limits. The method was optimized by selecting different strategies such as adding the triethylamine before the reagent and it yields goods results because it imparts the negative charge to the Oxygen of reacting phenol so that it can bind strongly with the reagent (10) . Also initially 500µl of mobile phase in order to dilute the sample, because the concentrated sample can block the column but the results were not good with 500µl mobile phase as some metabolites were unable to detect, so 50µl of mobile phase was used to dilute the sample.

5. Conclusion

The present study was conducted to test different chromatographic columns and thermo Orbitrap mass spectrometer for the efficient separation and detection of estrogens and their metabolites. The results suggested that the best resolution of estrogens and their metabolites and peak shapes were obtained by using C18-AR columns and Thermo Orbitrap mass spectrometer as compare to the other HPLC columns.

AUTHORS CONTRIBUTION STATEMENT

M. A: sample preparation and sample analysis, data interpretation, manuscript preparation

D. G. W: designing the project

M. I: data interpretation

M.S. A: data interpretation

T. I: manuscript preparation

A.S.P: manuscript preparation

A: manuscript preparation

Author contributions

All the authors contributed in the same proportion to the generation of this manuscript.

Declaration of interest

Authors declare that there is no conflict of interest.

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