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OPTIMIZATION OF DEPROTEINIZATION METHODS, HPLC METHOD DEVELOPMENT AND VALIDATION FOR QUANTIFICATION OF 6Β-HYDROXYTESTOSTERONE IN CELL CULTURE MEDIA

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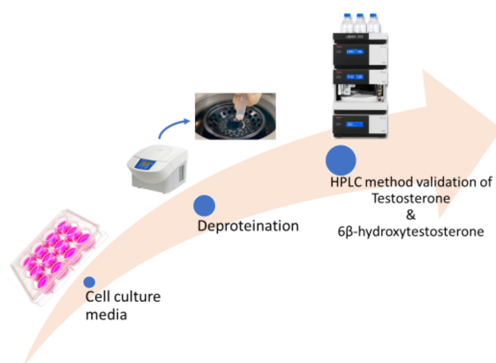
Graphical abstract



Abstract

Testosterone is commonly used as a marker probe drug for CYP3A4 metabolic activity. Accurate measurement of its metabolite, 6β-hydroxytestosterone in cell culture media is very crucial. This study aimed to optimize solvent extraction method as well as to develop and validate HPLC analytical method for the quantification of 6β-hydroxytestosterone. Testosterone and 6β-hydroxytestost-erone were extracted from culture media using different solvents and were analyzed using BCA assay and HPLC. The separation was performed at 0.7 mL/min flow rate, using gradient elution of water:methanol for 38 minutes at 242 nm. Solvent extraction of cell culture media using methanol showed the highest crude extract recovery, yield recovery of compounds, and percentage recovery of compounds and peak areas. Thus, the methanol extraction method was applied to further validate 6β-hydroxytestosterone HPLC analytical method. The specificity of the metabolite peak was excellent without any interference. The linear range was 0.156-5.000 ppm. The precision and accuracy were within acceptable criteria of <15%. Samples were stable at 4 °C chiller for up to 5 days, in autosampler for 24 hours, and in -20 °C freezer for up to one month. The method was successfully developed and validated for the quantification of 6β-hydroxytestosterone in cell culture media.

Keywords: 6β-Hydroxytestosterone, cell culture media, deproteinization, solvent extraction,method validation, HPLC

Abstrak

Testosteron seringkali digunakan sebagai substrat utama bagi eksperimen metabolisma enzim CYP3A4. Pengukuran tepat metabolitnya iaitu 6β-hydroxytestosterone di dalam media kultur sel adalah penting. Kajian ini bertujuan untuk mengoptimumkan kaedah pengekstrakan menggunakan pelarut dan validasi kaedah analisis HPLC untuk kuantifikasi 6β-hydroxytestosteron. Testosteron dan 6β-hydroxytestosterone telah diekstrak daripada media kultur dengan menggunakan pelarut yang berbeza. Seterusnya, ekstrak berkenaan dianalisis menggunakan ujian BCA dan HPLC. Pengasingan kromatografi dilakukan pada kadar aliran fasa bergerak ialah 0.7 mL/min dengan menggunakan elusi kecerunan air:metanol selama 38 minit pada 242 nm. Pengekstrakan media kultur sel menggunakan metanol menghasilkan ekstrak bahan mentah media, testosterone dan 6β-hydroxytestosterone tertinggi. Oleh itu, kaedah pengekstrakan metanol telah digunakan untuk mengesahkan lagi kaedah analisis HPLC bagi 6β-hydroxytestosterone. Keputusan pengasigan pada puncak kromatogram untuk metabolit tersebut adalah baik. Julat linear ialah 0.156-5.000 ppm. Kejituan dan kepersisan berada pada jumlah yang boleh diterima iaitu <15%. Ekstrak media stabil pada suhu 4 °C sehingga 5 hari, di dalam *autosampler* selama 24 jam dan di dalam peti sejuk pada suhu -20 °C bagi tempoh masa satu bulan. Kaedah ini berjaya dibangunkan untuk kuantifikasi 6β-hydroxytestosterone di dalam media kultur sel.

Kata kunci: 6β-Hydroxytestosterone, media kultur sel, menyah protein, ekstrak menggunakan pelarut, validasi kaedah, HPLC

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1.0 INTRODUCTION

Over the years, testosterone and 6β-hydroxytestosterone have gained interest as reference major chemical compounds to explore *in-vitro* metabolism-based study by Cytochrome P450 in cells culture. Testosterone well known as male sex hormone is a probe substrate to the liver enzyme CYP 3A4 subfamily of the Cytochrome P450 [1]. CYP3A4 enzyme present in the hepatocyte cells localized in smooth endoplasmic reticulum is responsible for catalyzing endogenous or exogenous testosterone through hydroxylation reaction to form more polar metabolites of 6β-hydroxytestosterone, then ready to be eliminated from the biological system [2]. The hydroxylated metabolites profile of testosterone can serve as the precursor for many studies against drug interaction, drug metabolism, toxicokinetic, metabolism mechanism and enzymology particularly using hepatic cells culture.

There are many liver-derived *in-vitro* systems that have been developed to assess the metabolism and potential toxic interactions of new chemical moiety, such as liver slices, primary and immortalized hepatocytes, liver microsomes and S9 fractions [3]. Previously, WRL68-CYP3A4 model was developed for evaluating CYP3A4 related drug interactions [4]. A well characterized CYP3A4 selective substrate is required to probe the activity of CYP3A4. Testosterone is one of the most relevant steroids that plays an essential role in the body [5]. Testosterone is a probe substrate biotransformed by the CYP3A enzymes in a regioselective and stereoselective manner into three major hydroxylated metabolites known as 6β-hydroxytestosterone, 2β-hydroxytestosterone, and 2a-hydroxytestosterone [2]. 6β-hydroxylation is one of the most commonly used metabolism assays for the assessment of CYP3A4 activity in human and other species [6].

Extraction of testosterone and 6β-hydroxytestosterone from cell culture media have been proposed as a primary step for accurate quantification of the compounds. Difficulty in analyzing both compounds in the extract mixed with other unknown compounds from the culture media may affect the accuracy of research findings. Plenty of previous study dealing with difference extraction and analytical methods strategies of the compounds in the cell culture media. For example, the culture media from transfected HepG2 cells with CYP3A4 DNA were extracted with dichloromethane and analyzed using high-performance liquid chromatography (HPLC) with isocratic mobile phase elution [7, 8]. Another study was carried out using methanol and acetone for testosterone extraction in the culture media obtained from the overexpress CPY3A4 enzyme in Caco-2/TC7 cells [9]. Then, the testosterone residue was examined by HPLC with gradient elution. There are more examples of extraction methods have been applied to extract the compounds in different *in-vitro* culture media from various types of cells or microsomals and using ordinary organic solvents, including acetone, acetonitrile, methanol, ethyl acetate and dichloromethane [10-14].

Although LC-MS has been proven to be superior in both sensitivity and specificity, identification and

quantification are possible using HPLC-DAD as well as an alternative tool [15]. The analytical analysis on the extraction of testosterone and 6β-hydroxytestosterone in cell culture media using various solvents with high amount of recovery for the compounds is still lacking. The interruption of remaining protein obtains from the cell culture media in HPLC analysis is unclear. Therefore, the present work aimed to investigate the optimum solvent extraction method of testosterone and 6β-hydroxytestosterone in WRL 68 cell culture media and to validate bioanalytical HPLC method for its quantification and analysis. The efficiency of various solvent extraction methods for protein depletion was also investigated. The data provided in the present study can be useful for improving metabolite extraction in cell culture media. This study also expected to be useful for the *in-vitro* cell-based evaluation of CYP3A4 activities by its metabolite (6β-hydroxytestosterone) quantification via HPLC-DAD analysis.

2.0 METHODOLOGY

2.1 Chemicals and Reagents

Testosterone, 6β-hydroxytestosterone, penicillin, and streptomycin were purchased from Sigma-Aldrich (St. Louis, MI, USA). Dulbecco's modified Eagle's Medium (high glucose), fetal bovine serum, TripLE™ select 1 × (no phenol), were purchased from (Gibco, Life Sciences). Methanol, acetonitrile, acetone, methanol, ethyl acetate, and dichloromethane were purchased from Merck. Thermo Scientific Hypersil™ ODS C_{18} column (4.6 x 100 mm, 3.5 µm).

2.2 Cell Counting and Treatment of Cell-Cultured Media

Normal human hepatic cells WRL 68 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's Medium (high glucose) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 24 hours. The passage number of the cells was maintained in the range of 19 to 40. The medium was replaced every two to three days until cells reached approximately 80% confluency and were passaged or used for experiments. The culture media was collected for analytical analysis

The cells were incubated for 24 hours at 37°C in a humidified atmosphere with 5% CO_2. The culture media in each well was added with testosterone and hydroxytestosterone to give a final concentration of 12 µM and 10 µM, respectively.

The experimental design included (a) fresh cell culture media, (b) cultured media without testosterone and 6β-hydroxytestosterone (c) cultured media containing testosterone and 6β-hydroxytestosterone. Each measurement determination was conducted in three independent experiments.

2.3 Extraction of Testosterone and 6β-hydroxytestos-Terone from Cell-cultured Media

The cultured media containing testosterone and 6β-hydroxytestosterone were collected. Extraction of the compounds using different solvents including acetone, acetonitrile, and methanol were conducted separately by gentle shake mixing 250 µL cell culture media with 1 mL solvent for 5 minutes and centrifuging for 20 minutes at 14000 rpm. While for solvent extraction using ethyl acetate and dichloromethane, 250 µL of the cell culture media and 1 mL solvent were mixed with gentle shake for 5 minutes, centrifuged for 15 minutes at 3000 rpm, and the organic layer was collected. Solvents were evaporated to dryness under nitrogen. The protein content of the residual was analyzed using Bichinconinic acid assay (BCA) method and HPLC for quantification of testosterone and 6β-hydroxytestosterone.

2.4 Protein Quantification Assay

Protein concentrations from the dried residual were quantified according to the method established by Bradford (1976) using bovine serum albumin as a standard. The residual was dissolved in 150 µL of 5% methanol and was mixed homogenously with 1.25 mL Protein Reagent Blue G-250. The mixture was incubated for 30 minutes at 30°C, and the absorbance of the mixture was measured at 595nm. Standard bovine serum albumin was dissolved in 5% methanol at concentrations from 62.5 to1000 µg/mL. The standard linear curve of standard bovine serum albumin was plotted to quantify total protein concentrations in the sample dried residual in each solvent extraction method.

2.5 Quantification of Testosterone and 6β-Hydroxytes-tosterone using HPLC

Separation using HPLC was performed on Dionex Ultimate 3000 Quaternary Pump that equipped with degasser, temperature controlled automated liquid sampler, temperature-controlled column compartment, diode-array detector and ChemStation software used as interface data processing (Thermo Fisher Scientific Inc., MA, USA). Chemical profiling of the extract was carried out by gradient elution system of deionized water (A) and methanol (B); 5% B (0-1.25 min, 5-95% B (1.25-25 min), 95% B (25-30 min), 95-5% B (30-33 min) and 5% B (33-38 min) using Thermo Scientific HypersilTM ODS C18 column; 4.6 x 100 mm, 3.5 µm (Thermo Fisher Scientific, USA) at 28 °C of temperature and 0.7 mL/min of flow rate. A volume of 10 µL of sample was injected into the column at concentration 10 mg/mL and observed at 242 nm of wavelength. 6β-

hydroxytestosterone from testosterone 6β-hydroxylation activity of WRL68-CYP3A4 system was calculated by referring to a standard curve constructed based on known six-point serial dilution at concentrations ranging from 0.156 to 5.000 µg/mL.

2.6 HPLC Method Validation

The HPLC methods for quantification of 6β-hydroxytestosterone was validated in accordance with the ICH Harmonised Tripartite Guideline Q2 (R1) (ICH 2005).

2.6.1 Specificity

The specificity of the methods was evaluated using 6 batches of blank culture medium.

2.6.2 Linearity, Limit of Quantification (LOD), and Limit of Detection (LOQ)

The calibration curve consists of six concentrations 0.156, 0.313, 0.625, 1.250, 2.500, and 5.000 µg/mL of analyte standards (6β-hydroxytestosterone or 4'-hydroxydiclofenac) spiked into culture media. On 3 different days, each concentration level was prepared and assayed in technical triplicates. Slope, intercept, and correlation coefficient were determined. The equations used for calculations were; LOD = (3.3 x σ)/ slope, and LOQ = (10 σ)/ slope. σ defined as standard deviation of the lowest point.

2.6.3 Precision and Accuracy

Intra-day and inter-day precision and accuracy were determined by conducting QC samples at low, medium, and high concentrations (0.31, 1.25, and 5.00 µg/mL) in triplicates on the same day and on 3 different days. Precision was expressed as the relative standard deviation (RSD), the equation used was; RSD = (standard deviation *100) / mean. On the other hand, accuracy was evaluated by comparing the measured concentration with the true value. Criteria of acceptance is the deviation is within ± 20% for low concentration QC samples and not more than 15% for QC samples with medium and high concentration.

2.6.4 Recovery

The recovery of analyte extraction was determined by comparing the peak areas from culture media samples spiked before extraction with the peak areas from the ones spiked after extraction. The equation used to calculate percent recovery was; percent recovery = (peak area of extracted metabolite/ peak area of spiked metabolite without extraction) x 100%.

2.6.5 Stability

The stability of the analytes culture media sample was assessed by evaluating the QC samples stored in 4°C chiller for 24 hours, overnight in an autosampler at temperature of 30°C , and in a -20°C freezer for 1 months. QC samples at low and high concentrations were analysed in triplicates for each storage condition.

2.7 CYP3A4-mediated Testosterone 6β-Hydroxylation Assay

Transiently transfected WRL68 cells overexpressing CYP3A4 (WRL68-CYP3A4) with 80% confluency in 6 well plate were treated with various concentration of substrate testosterone (25 to 250 µM). Concentration of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) was not more than 0.01% in the treatment. After incubation for 2.5 hours, the media was collected and processed using optimized method to extract the metabolite 6β-hydroxytestosterone. A volume of 250 µL of media was added with 1 mL methanol before vigorous shaking for 5 minutes and then was centrifuged at 14000 rpm for 15 minutes. Supernatant was transferred to a new tube and evaporated to dryness under nitrogen gas stream. Evaporated samples were reconstituted in 150 µL of 5% methanol prior to injection into the HPLC system.

2.8 Statistical Analysis

Experimental data were expressed as mean ± standard deviation (SD) with the number of independent experiments in triplicates (n = 3). Analysis was carried out using software of Microsoft excel and SPSS 21. Differences between the values of various experimental groups were assessed with one-way analysis of variance (ANOVA) with the Posthoc test of Tukey, student's t-test, and a P value less than 0.05 was indicated to be statistically significant.

3.0 RESULTS AND DISCUSSION

3.1 Crude Protein Content by Different Extraction Methods

The protein content in various extracts of WRL 68 cell culture media were quantified using BCA assay. The protein content in 250 mL culture media incubated for 24 hours with WRL 68 cells was used as reference for protein depletion percentage calculation. Table 1 showed protein content in all solvent extracts, which ranged from 11.82 to 231.12 µg with percentage depletion ranging from 98.82 to 76.80%. The protein content in the culture media extracted using ethyl acetate was significantly lower (P < 0.05) at 11.82 µg (98.82% depletion), followed by dichloromethane extract at 86.92 µg of protein (91.27% depletion). This indicates that among the solvents used, ethyl acetate is the best and most efficient for the purpose of removing protein in the culture media.

Table 1 Protein obtained from different extraction methods of metabolites in WRL68 cell culture media using Bicinchoninic Acid protein assay

Extraction method	Protein content (µg)	Protein depletion (% w/w)
Acetone	^{c,d,e} 231.12 ± 7.87	^{c,d,e} 76.80 ± 1.56
Acetonitrile	^{d,e} 221.20 ± 4.54	^{d,e} 77.81 ± 0.87
Methanol	^{a,d,e} 199.87 ± 18.29	^{a,d,e} 79.95 ± 1.87
Ethyl acetate	^{a,b,c,e} 11.82 ± 0.52	^{a,b,c,d} 98.82 ± 0.03
Dichloromethane	^{a,b,c,d} 86.92 ± 5.43	^{a,b,c,d} 91.27 ± 0.87

Protein concentration in each solvent extraction method was determined by the internal standard of serum bovine albumin at concentration ranging from 1000 to 0 µg/mL (n=3). The protein content was determined in 250 µL cultured media. Cell cultured media without extraction method was set as reference with protein content 997.76 ± 42.89 µg and was extrapolated to calculate protein depletion. ^{a} Represents the highest protein content among the extraction methods. ^{b} Represents the lowest protein depletion among the extraction methods. Data are given as mean ± SD (n=3). ^{a} $P < 0.05$ vs acetone, ^{b} $P < 0.05$ vs acetonitrile, ^{c} $P < 0.05$ vs methanol, ^{d} $P < 0.05$ vs ethyl acetate, ^{e} $P < 0.05$ vs dichloromethane (one-way anova)

The protein could be from the dead cell, serum albumin, enzymes and metabolism products [16-18]. Protein removal is an important step in sample preparation to ease the analysis of secondary metabolites in culture media. Ethyl acetate and dichloromethane extraction were able to deplete protein content greater than 90%. Since the majority of the proteins in the media culture are polar, extraction with the less polar solvents like ethyl acetate and dichloromethane minimized protein transfer to the solvent [19, 20]. The results are in line with the interference free peaks in the chromatograms (Figure 1). More polar proteins can be observed in the chromatograms of acetone, acetonitrile and methanol extract. However, it should be noted that signals for the secondary metabolite of interest in this work which are testosterone and 6β-hydroxytestosterone were not were affected in the chromatogram. Previous studies demonstrated that the polar solvents succeeded to extract higher amounts of proteins compared to non-polar solvents [19, 20]. However, the protein contents in the solvent extracts did not interrupt the qualitative and quantitative analysis of testosterone and 6β-hydroxytestosterone.

3.2 Qualitative and Quantitative Analysis of Testosterone and 6β-hydroxytestosterone

Various solvent extraction methods of testosterone and 6β-hydroxytestosterone in WRL 68 cell culture media were analyzed using HPLC. The solvents included acetone, acetonitrile, methanol, ethyl acetate, and dichloromethane individually tested to recover the yield contained both compounds in the culture media. The use of different solvents in the extraction of the compounds from the cell culture media affected the extract contents and led to a challenge in HPLC analysis. The challenge involves the separation of chromatogram peaks between the targeted compounds and unidentified peaks present in the media. HPLC analysis of testosterone and 6β-hydroxytestosterone is well established in many *in vitro* metabolism-based study as method of choice that varies to elution techniques either isocratic or gradient [7, 21-23].

Based on the optimum HPLC conditioning, the present study successfully resolved separation peaks of testosterone and 6β-hydroxytestosterone from other contaminants for all solvent extracts as shown in Figure 1. The peaks of both compounds in all solvents extracted were symmetry and narrow peak width. The chromatogram showed the HPLC condition of mobile phase methanol:water with gradient elution produced a good separation between the known compound peaks and contaminant peaks from the culture media. The separation of the targeted compounds achieved satisfactory when using gradient elution due to interference from unknown chemical constituents in the culture media, and isocratic elution was not applicable in the HPLC analysis [24, 25]. The contaminants from the culture media were more polar than the targeted compounds which rapidly eluted without separation. Almost similar separation pattern of testosterone, hydroxytestosterone and contaminants in the cell culture media DMEM "High Glucose" and Chinese hamster ovary cell culture media were observed in the HPLC chromatogram of previous results [23, 26]. The unresolved peaks of the polar chemical constituents obviously demonstrated in the chromatograms of acetone, acetonitrile and methanol extracts (Figure 1). Solvents extraction methods of ethyl acetate and dichloromethane produced chromatograms with less contaminant peaks. The polarity of solvent and extraction technique could be the factors of contribution to the extraction or contaminants from the culture media.

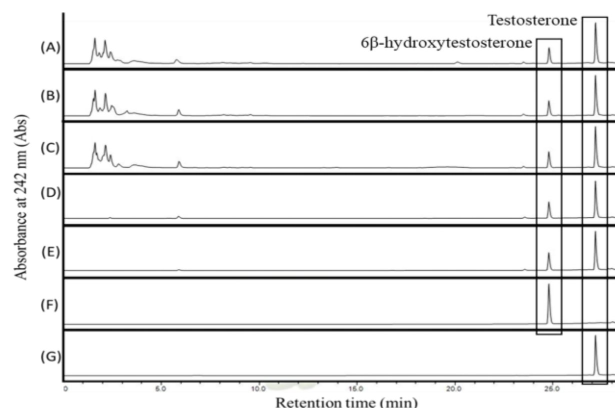

Figure 1 Chromatograms of testosterone and 6β-hydroxytestosterone obtained from difference solvent extraction methods. (A) represents chromatogram of acetone extraction method, (B) represents acetonitrile extraction method, (C) represents chromatogram of methanol extraction method, (D) represents ethyl acetate extraction method, (E) represents chromatogram of dichloromethane extraction method, (F) represents chromatogram of 6β-hydroxytestosterone and (G) represents chromatogram of testosterone

The peak area of testosterone and 6β-hydroxytestosterone from the chromatograms for different solvent extracts were summarized in Figure 2. The highest recovery of both compounds as determined by the peak areas was demonstrated when solvent extraction was conducted with methanol and revealed statistical significant difference ($P < 0.05$) value of the peak areas in comparison with each solvent extract. Ethyl acetate solvent extract was determined significantly lower ($P < 0.05$) of peak areas for the compounds. The peak areas of acetone, acetonitrile and dichloromethane did not show statistically significantly different ($P > 0.05$) for both compounds, testosterone and 6β-hydroxytestosterone.

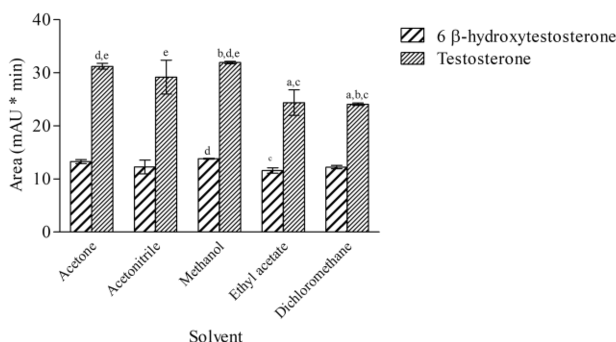

Figure 2 Chromatogram peak areas of testosterone and 6β-hydroxytestosterone discovered from difference solvent extraction methods. Each data represents as mean ± SD (n=3). [a] $P < 0.05$ vs acetone, [b] $P < 0.05$ vs acetonitrile, [c] $P < 0.05$ vs methanol, [d] $P < 0.05$ vs ethyl acetate, [e] $P < 0.05$ vs dichloromethane (one-way anova)

3.3 Extraction Yield of Crude Extract, Testosterone and 6β-hydroxytestosterone

The yield of crude extracts from 250 µl WRL 68 cell culture media spiked with 0.8651 mg of testosterone and 0.7848 mg 6β-hydroxytestosterone, obtained by different types of solvent extraction methods were calculated as shown in Table 2. The weight of yield extractable by the solvents ranged from 4.88 mg to 0.80 mg. Among the solvents extraction conducted, methanol demonstrated significant higher ($P < 0.05$) yield extract (4.88 mg) followed by solvent extraction method of acetone (4.54 mg), acetonitrile (4.23 mg), dichloromethane (3.52 mg) and ethyl acetate (0.80 mg) demonstrated the lowest weight ($P < 0.05$) of yield extract.

The peak areas of testosterone and 6β-hydroxytestosterone for each solvent extract were extrapolated to determine yield recovery and percentage recovery (Table 2). The results revealed that methanol extraction showed significant higher ($P < 0.05$) yield recovery of testosterone (0.85 mg, 98.30%) and 6β-hydroxytestosterone (0.77 mg, 98.39%) compared to other solvent extraction methods. In addition, the yield recovery and percentage recovery results of both compounds by the methanol extraction were parallel with the significant higher ($P < 0.05$) peak area findings. Acetone also showed high recovery in the extraction of testosterone with yield extract 0.83 mg (96.07%), and for yield extract of 6β-hydroxytestosterone was 0.74 mg (94.56%). The next highest ($P < 0.05$) yield recovery was acetonitrile extraction resulted testosterone yield 0.78 mg (89.64%) and 6β-hydroxytestosterone yield 0.69 mg (94.56%). However, significant low ($P < 0.05$) yield recovery of testosterone and 6β-hydroxytestosterone were obtained in the ethyl acetate and dichloromethane extracts compared with methanol, acetone and acetonitrile.

Table 2 Concentration of testosterone and 6β-hydroxytestosterone recovered from cell culture media using different solvent extraction methods

Solvent extraction method	Yield extract (mg)	Yield recovery (µg)		Percentage recovery (% w/w)	
		Tes*	6β-OHtes**	Tes*	6β-OHtes**
Acetone	4.54 ± 0.17	[d,e] 0.83 ± 0.01	[d,e] 0.74 ± 0.02	[d,e] 96.07 ± 1.71	94.56 ± 2.63
Acetonitrile	4.23 ± 0.12	[e] 0.78 ± 0.09	[c,d,e] 0.69 ± 0.07	[e] 89.64 ± 10.10	87.47 ± 8.93
Methanol	[c] 4.88 ± 0.07	[d,e] 0.85 ± 0.01	[b,d,e] 0.77 ± 0.01	[d,e] 98.30 ± 0.46	[d] 98.39 ± 0.58
Ethyl acetate	[d] 0.80 ± 0.07	[a,c] 0.64 ± 0.07	[a,b,c,e] 0.65 ± 0.03	[a,b,c] 74.49 ± 7.59	[c] 82.98 ± 3.59
Dichloromethane	3.52 ± 0.30	[a,b,c] 0.64 ± 0.01	[a,b,c,d] 0.69 ± 0.02	[a,b,c] 73.54 ± 0.74	[a] 87.31 ± 2.18

*Tes refers to testosterone. **6β-OHtes refers to 6β-hydroxytestosterone. Yield recovery in each solvent extraction method was determined by the internal standard of testosterone and 6β-hydroxytestosterone at concentration ranging from 10 to 0.15625 ppm (n=3) with the linear equation for testosterone was $y = 7.738x - 2.158$ and $r^2 = 0.9758$ and 6β-hydroxytestosterone was $y = 2.407x + 0.05164$. Yield recovery was analyzed in 250 µL cultured media spiked with testosterone and 6β-hydroxytestosterone. Percentage recovery was calculated by comparing the yield recovery with the theoretical total concentration of testosterone and 6β-hydroxytestosterone spiked in the 250 µL cell culture media with the value 0.8651 µg and 0.7848 µg, respectively. Each data represents as mean5 ± SD (n=3). [a] $P < 0.05$ vs acetone, [b] $P < 0.05$ vs acetonitrile, [c] $P < 0.05$ vs methanol, [d] $P < 0.05$ vs ethyl acetate, [e] $P < 0.05$ vs dichloromethane (one-way anova).

The HPLC analysis of testosterone and 6β-hydroxytestosterone provided consistent retention times and good measurable separation results of peak areas. Individual peak area of the compounds determined in difference solvent extracts were various values. Methanol and ethyl acetate extracts showed significant increase and decrease of the peak areas for the compounds, respectively. The findings were consistent with the total yield crude extract and recovery amount of the testosterone and 6β-hydroxytestosterone. Optimum extraction of the compounds content as measured by the HPLC peak areas was obtained when extractions were performed with methanol solvent that showed high percentage recovery for both compounds more than 98%. On the other hand, the lowest recovery of the compounds showed by solvents extraction from ethyl acetate and dichloromethane with percentage recovery ranging from 74.49% to 87.98%. Present study suggests that testosterone and 6β-hydroxytestosterone in the WRL 68 cell culture media is easier to extract with more polar solvent especially by methanol known as the highest polarity among the other solvents. The present of hydroxyl group in

the chemical structure of methanol possibly increase the chemical interactions between solvent and solute (testosterone and 6β-hydroxytestosterone).

3.4 Validation of the HPLC Quantitative Analysis of 6β-hydroxytestosterone

In this study, HPLC quantification method for 6β-hydroxytestosterone as the main metabolite produced from CYP3A4 mediated testosterone metabolism was further validated in accordance with the ICH Harmonised Tripartite Guideline Q2 (R1) (ICH 2005). Specificity, linearity, limit of quantification (LOQ), limit of detection (LOD), precision, accuracy, extraction recovery and stability parameters of the method was assessed, and the results were summarized in Table 3.

In a validation procedure, specificity parameter should confirm the ability of the method to unequivocally assess the analyte in the presence of other components such as impurities and degradation products, that may be present in the matrix [27]. Specificity was evaluated by comparing chromatograms of batches of blank media and the ones spiked with analyte of interest. Based on the result shown in Figure 3, the retention times of 6β-hydroxytestosterone was 24.8 min and there is no interference from blank media observed.

The linearity refers to the ability of an analytical method to derive results that are directly proportional to the concentration of the analyte within a given range in the sample [28]. The linear range was 0.156-5.000 µg/mL resulted in calibration plots with high linearity (R^2> 0.99) as shown in Figure 4. The detection and quantification limits of the method was lower than 1 µg/mL indicating that the method enable sufficient sensitivity for quantification of respective analytes.

The ICH guideline defines precision as the closeness of agreement or degree of scatter between a series of measurements obtained from multiple sampling of the same homogenous sample using prescribed protocol and conditions. On the other hand, the accuracy or also termed as trueness, refers to the closeness of agreement between the true value or a reference value and the value measured [29]. The precision and accuracy of the method was determined within a single day and between three different days using low, medium and high quality control (QC) concentrations of the analytes (0.31, 1.25 and 5.00 µg/mL), each sample were prepared in triplicates following the recommendation by ICH guideline. Precision was expressed as the relative standard deviation (RSD), while the accuracy expressed as a percent of nominal in which evaluated by comparing the measured concentration with the true value.

Table 3 Validation parameters for quantification of 6β-hydroxytestosterone using HPLC-DAD

Parameters	Values		
Linear range (µg/mL)	0.156-5.000		
Equation	y = 4.4427 +0.0459		
Coefficient of determination, R^2	0.9941		
Limit of detection, LOD (µg/mL)	0.0087		
Limit of quantification, LOQ (µg/mL)	0.0290		
Specificity	No interference		
Nominal QC concentrations (µg/mL)	**0.31**	**1.25**	**5.00**
Intra-day precision for QC samples (%RSD)	2.66	1.02	2.18
Intra-day accuracy for QC samples (%)	95.99	102.57	97.56
Inter-day precision for QC samples (%RSD)	2.37	3.62	2.99
Inter-day accuracy for QC samples (%)	95.29	96.56	95.39
Extraction recovery (%)	99.69 (%RSD = 1.48)	92.46 (%RSD = 1.84)	96.97 (%RSD = 5.06)
Stability at 4°C chiller for days (%)	96.06 (%RSD = 5.75)	-	94.62 (%RSD = 2.29)
Stability in autosampler for 24 hours (%)	93.07 (%RSD = 3.31)	-	98.35 (%RSD = 0.93)
Stability at -20 °C freezer for 1 month (%)	99.10 (%RSD = 1.37)	-	99.80 (%RSD = 4.62)

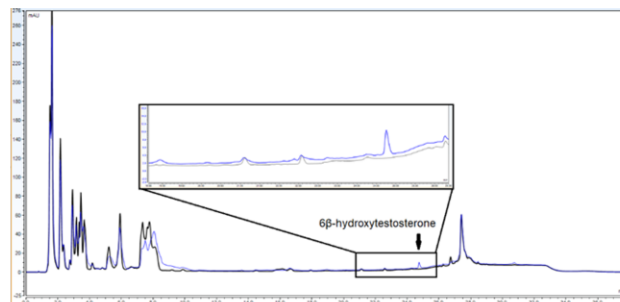

Figure 3 Specificity of 6β-hydroxytestosterone peak. HPLC chromatograms overlain of blank media (black) and spiked with 0.156 µg/mL 6β-hydroxytestosterone (blue), retention time=24.8 min, λ=242nm. No peak interference observed

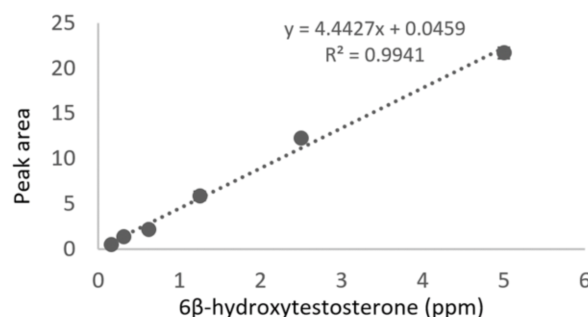

Figure 4 Standard curve of 6β-hydroxytestosterone (top) showing high linearity (R^2> 0.99)

The intraday accuracy results ranged between 97.00 and 103.10% with precision of (RSD) 2.89-4.20%, while the interday accuracy ranged from 94.5 to 97.9% with precision of (RSD) 1.02-2.81%. The developed method exhibits good precision and accuracy for low, medium and high QC sample, which fall within acceptable criteria.

The recovery measures the closeness between the concentration obtained after a spiked sample undergone extraction and preparation processes to the true concentration, and 100% recovery considered as the best value [30]. For this method, the sample preparation for analyte 6β-hydroxytestosterone involved protein precipitation technique where methanol was added to the media as precipitating agent. The recovery of the analyte in low, medium and high QC samples was greater than 90%.

Stability test was carried out to find out the stability of the analytes in different short-term and long-term storage conditions. The analyte, 6β-hydroxytestosterone in low and high QC samples were found stable in three different storage conditions in autosampler for 24 hours, 4°C chiller for 5 days, and -20 °C freezer for 1 month. In this study, samples were routinely injected into HPLC system within 12 hours after preparation. No samples were stored on the bench due to the fluctuating room temperature in the labs. Prepared samples were immediately stored in 4°C chiller before transferred to autosampler for analysis. For long-term storage in -20°C freezer, samples were only thawed once and never undergone freeze-thaw cycles, samples were stored up to 1 month and not longer. This is to make sure that no samples were stored in conditions other than that have been validated in this study.

Figure 5 illustrates detection of 6β-hydroxytestosterone formation after enzymatic reactions using WRL68-CYP3A4 system. Although small interference was observed on testosterone peak in Figure 5(D), it does not affect the quantification of analyte 6β-hydroxytestosterone. Therefore, the method was successfully validated for quantification of mentioned analytes.

4.0 CONCLUSION

The solvent extraction methods of acetone, acetonitrile, methanol, ethyl acetate, and dichloromethane were compared for the HPLC analysis of testosterone and 6β-hydroxytestosterone in cell culture media. The HPLC optimum condition water:methanol mobile phase with the application of gradient elution successfully resolves chromatogram peaks of the targeted compounds from chemical components of cell culture media. In addition, the HPLC analysis was not affected by the protein content of the cell culture media. The use of methanol in the solvent extraction of cell culture media is practically efficient in obtaining high recovery of testosterone and 6β-hydroxytestosterone.

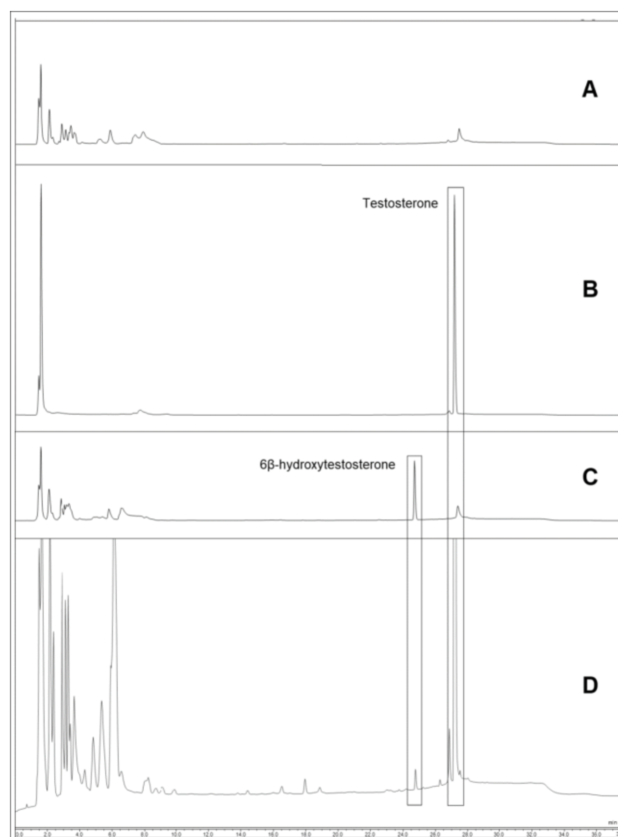

Figure 5 UV–HPLC chromatograms of testosterone 6β-hydroxylation by WRL68-CYP3A4 model. (A) blank media, (B) testosterone peak shown at t_R = 27.1 min, (C) 6β-hydroxytestosterone peak shown at t_R = 24.8 min, (D) Product 6β-hydroxytestosterone after 150 min incubation with testosterone (200 μM). Monitoring at λ= 242 nm

The sample extracted using methanol contains the highest amount of protein compared to other solvents. The solvent extraction methods with minimum protein content can be obtained primarily by ethyl acetate extraction followed by dichloromethane. The recovery of testosterone and 6β-hydroxytestosterone in the culture media is not comparable with the methanol extraction method. The optimized and validated bioanalytical method for measuring 6β-hydroxytestosterone in cell culture media using HPLC-DAD is useful as a tool for the *in vitro* cell-based evaluation of CYP3A4 activity.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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