Development of an analytical method to measure 17β-estradiol metabolite concentrations in MCF-7 and MCF-12A cell lines.

by

Hermia van Zyl (9700538)

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Study leaders: Dr M-L Lottering

Dr A Joubert

Dr JB Laurens

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This project is dedicated to my Lord God: Father, Son and Holy Spirit. For without His mercy, grace and continuous encouragement and strengthening, I would not have been able to complete what I have started

Numbers 23:19

God is not a man, that He should tell or act a lie, neither the son of man, that He should feel repentance or compunction [for what He has promised]. Has He said and shall He not do it? Or has He spoken and shall He not make it good?

Hebrews 10: 35-36

Do not, therefore, fling away your fearless confidence, for it carries a great and glorious compensation of reward. For you have need of steadfast patience and endurance, so that you may perform and fully accomplish the will of God, and thus receive and carry away [and enjoy to the full] what is promised.

Philippians 2: 14

Do all things without grumbling and faultfinding and complaining [against God] and questioning and doubting [among yourselves],

Philippians 4:13

I have strength for all things in Christ who empowers me [I am ready for anything and equal to anything through Him Who infuses inner strength into me; I am self-sufficient in Christ's sufficiency].

1 Corinthians 10:13

… But God is faithful [to His word and His compassionate nature], and He [can be trusted] not to let you be tempted and tried and assayed beyond your ability and strength of resistance and power to endure, but with the temptation He will [always] also provide the way out (the means of escape to a landing place), that you may be capable and strong and powerful to bear up under it patiently.

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Abstract

Breast cancer is one of the most common cancers affecting women. It remains the leading cause of death in American women from 30 – 70 years of age and approximately 10% of the women living in western countries will develop breast cancer during their life time. Estrogens are a family of female hormones involved in the reproductive function of the human body. Estradiol is the most abundant estrogen in premenopausal woman. Initially it was thought that estradiol itself was responsible for tumourigenesis, but it has since been discovered that the catechol metabolites of estradiol and other estrogens cause carcinogenesis. 4-Hydroxyestradiol (4-HE_2) is a potent cell proliferating estrogen whereas 2-methoxyestradiol (2-ME_2) is a potent inhibitor of cell proliferation through the activation of apoptosis. 2-Hydroxyestradiol (2-HE_2) also causes increased cell proliferation but it is not as potent as 4-HE_2 and it is *O*-methylated rapidly to 2-ME_2. Catechol metabolites of estradiol are also involved in producing reactive oxygen species through redox cycling. The reactive oxygen species cause DNA damage and mutations to occur which can lead to carcinogenesis. A significant ratio to consider as a biomarker for breast cancer risk therefore is the 4-HE_2/2-ME_2 ratio. In this study, an analytical method was developed to measure the concentration levels of E_2, 2-ME_2, 2-HE_2 and 4-HE_2 in cell culture medium. The analytical method made use of gas chromatography-mass spectrometry (GCMS) analysis, since the expected physiological concentrations of these metabolites were very low. Various extraction and derivatisation techniques were applied during the development of the method. The final method made use of protein precipitation with concentrated hydrochloric acid, liquid-liquid extraction using diethyl

ether and derivatisation with trimethylsilylimidazole (TMSI). During the validation of this method, it was found that the method did not produce accurate measurements and that it could only be used to determine trends. Since the precise *in vitro* concentration levels of the metabolites were still unknown, it was decided to proceed with experiments using this method, to provide preliminary results with which further course of action could be planned. Equal numbers ($1x10^6$ cells/flask) of MCF-7 and MCF-12A cells were provided with 11 ml medium containing E_2/2-HE_2/4-HE_2/2-ME_2 (10^{-6} M) and medium containing E_2/2-HE_2/4-HE_2/2-ME_2 (10^{-8} M). Of each metabolite and each concentration two flasks were prepared. Each flask represented a specific time interval. At the appropriate time 10 ml of the medium was extracted. The time intervals used for each experiment were 0 hours, 8 hours and 24 hours for incubation with E_2, 0 hours, 1 hour and 8 hours for incubation with 2-ME_2, 0 hours, 10 minutes and 1 hour for incubation with 2-HE_2 and 4-HE_2. The time intervals used were according to the expected rate of metabolism. Each experiment was repeated three times. Differences in the metabolism of breast tumour cells and normal cells were found and the concentration of the metabolites present in the cell incubation medium had an influence on the metabolism of the cells. The need to investigate the intracellular concentrations of the metabolites has also been accentuated through the results obtained.

Opsomming

Borskanker is een van die mees algemene vorme van kanker wat by vrouens voorkom. Dit bly die vernaamste oorsaak van dood onder vrouens tussen 30 en 70 jaar en ongeveer 10% van die vrouens in westerse lande sal borskanker ontwikkel gedurende hulle leeftyd. Estrogene is 'n familie van vroulike hormone wat betrokke is by die voortplantingsfunksie van die menslike liggaam. estradiol is die vollopste estrogeen in premenopousale vrouens. Oorspronklik is daar gedink dat estradiol self verantwoordelik was vir die tumorgenese maar intussen is vasgestel dat die katesjolmetaboliete van estradiol karsinogenese veroorsaak. 4-Hidroksie estradiol (4-HE_2) is 'n sterk selprolifererende estrogeen terwyl 2-metoksie estradiol (2-ME_2) 'n sterk inhibitor is van selproliferasie deur die aktivering van apoptose. 2-Hidroksie estradiol (2-HE_2) veroorsaak ook verhoogde selproliferasie, maar tot 'n mindere mate as 4-HE_2 en dit word vinnig ge-*O*-metileer na 2-ME_2. Die katesjolmetaboliete van estradiol is ook betrokke by die vorming van reaktiewe suurstofspesies deur die redokskringloop. Die reaktiewe suurstofspesies veroorsaak DNS skade en mutasies wat kan lei tot karsinogenese. 'n Vername verhouding om ingedagte te hou as 'n biomerker vir borskankerrisiko is die 4-HE_2/2-ME_2 verhouding. In hierdie studie is 'n analitiese metode ontwikkel om die konsentrasievlakke van E_2, 2-ME_2, 2-HE_2, en 4-HE_2 in selkultuurmedium te meet. Die metode maak gebruik van gaschromatografie-massaspektrometrie (GC-MS) analise omdat die verwagte fisiologiese konsentrasies van hierdie metaboliete baie laag is. Verskeie ekstraksie- en derivatiseringstegnieke is beproef tydens die ontwikkeling van die metode. Die finale metode het gebruik gemaak

van proteïenpresipitasie met gekonsentreerde soutsuur, vloeistof-vloeistof ekstraksie met diëtieleter en derivatisering met trimetielsililimidazool (TMSI). Gedurende die validering van die metode is daar gevind dat die metode nie akkurate metings produseer nie en dat dit slegs gebruik kan word vir die bepaling van nygings. Omdat die presiese *in vitro* konsentrasievlakke van die metaboliete nog nie bekend is nie, is daar besluit om voort te gaan met eksperimente en gebruik te maak van hierdie analitiese metode om resultate te produseer waarmee die rigting vorentoe kan beplan word. Gelyke hoeveelhede ($1x10^6$selle/fles) van MCF-7 en MCF-12A selle was voorsien van 11 ml medium wat E_2/2-HE_2/4-HE_2/2-ME_2 (10^{-6} M) en medium wat E_2/2-HE_2/4-HE_2/2-ME_2 (10^{-8} M) bevat. Vir elke metabioliet en elke konsentrasie is twee flesse voorberei. Elke fles het ‘n spesifieke tydsverloop verteenwoordig. Na afloop van die korrekte tydsinterval is 10 ml van die medium afgetrek. Die betrokke tydsintervalle was 0, 8 en 24 uur vir E_2, 0, 1 en 8 uur vir 2-ME_2, 0, 10 minute en 1uur vir 2-HE_2 en 4-HE_2. Die tydsintervalle was bepaal volgens die verwagte tempo van metabolisme. Elke eksperiment was drie keer herhaal. Verskille in die metabolisme van borskankerselle in vergelyking met normale selle is gevind en die konsentrasies van die metaboliete teenwoordig in die selinkubasiemedium het ‘n invloed op die metabolisme van die selle. Die behoefte aan verdere navorsing in hierdie gebied het ook na vore gekom uit die verkrygde resultate.

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Abbreviations

•OH	hydroxyl radical
16α-HE_1	16α-hydroxyestrone
16α-HE_2	16α-hydroxyestradiol
17β-HSD	17β-hydroxysteroid dehydrogenase
2-HE_2	2-hydroxyestradiol
2-ME_2	2-methoxyestradiol
4-HE_2	4-hydroxyestradiol
4-HE_2-d_5	deuterated 4-hydroxyestradiol
5β-C	5β-cholestane
B	longitudinal diffusional spreading
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
C4	carbon atom 4
CE	catechol estrogens
C_m	resistance to mass transfer in the mobile phase
COMT	catechol *O*-methyl transferase
C_s	resistance to mass transfer in the stationary phase
CV	coefficient of variation
CYP450	cytochrome P450
DEE	diethylether
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid

E_1	estrone
E_2	estradiol
E_3	estriol
ECD	electron capture detection
EI	electron impact
EIC	extracted ion chromatogram
FID	flame ionisation detection
FSH	follicle-stimulating hormone
GC	gas chromatography
GC-FID	gas chromatography with flame ionisation detection
GC-MS	gas chromatography – mass spectrometry
GSH-S-transferase	glutathione S-transferase
H	theoretical plate height
HCl	hydrochloric acid
HPLC	high pressure liquid chromatography
i.d.	internal diameter
kPa	kilopascal
LH	luteinising hormone
MCF-12A	Michigan Cancer Foundation cell line 12A
MCF-7	Michigan Cancer Foundation cell line 7
MEME	minimum essential medium eagle
MS	mass spectrometer
MTBSTFA	N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide

N	number of theoretical plates
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
$O_2^{\bullet-}$	superoxide radical
PBS	phosphate buffered saline
ppb	parts per billion
ppm	parts per million
ppt	parts per trillion
RIA	radioimmuno assay
RNA	ribonucleic acid
ROS	radical oxygen species
R_s	resolution
SCOT	support coated open tubular
SIM	single ion monitoring
t-BDMS	t-buthyldimethylsilyl
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TDLU	terminal ductal lobular units
TIC	total ion chromatogram
TMS	trimethylsilyl
TMSI	trimethylsilylimidazole
WCOT	wall coated open tubular