

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

by

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**Submitted in fulfilment of part of the requirements for the degree of
Master of Science (Physiology) in the Faculty of Health Sciences
University of Pretoria**

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November 2004

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.

Acknowledgements

I would like to thank the following:

- My Lord God: Father, Son and Holy Spirit, who are my source of strength and hope.
- My mother and father, for their encouragement and belief in me.
- My brothers, for their interest in my work and their love displayed in my life.
- My dear friend Hanri, for coping with my mood swings and frustration and still remaining a true friend.
- My dear friend Vera, for her support from afar.
- Dr Mona-Liza Lottering, for her excellent leadership, and continual support.
- Dr Annie Joubert, for her emotional support and leadership.
- Dr Tim Laurens, for his patience with my lack of knowledge and for showing me what it is to be a researcher.
- Dr Ilse Ker, for her friendship and for listening when I needed to blow off some steam.
- Dr Becker from the Medical Research Council for his help with the statistical planning of the physiological side of this study.
- The Department of Chemical Pathology at the University of Pretoria, for making their labs and instrumentation available to me, and for excepting me as one of their own.
- The Department of Physiology at the University of Pretoria, for making their labs and instruments available to me, and supporting me throughout this project.
- Marie Griffiths, who lighted the candle of interest and love for this incredible subject of Physiology.
- Angelique Elliott, for checking my grammar.

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-HE₂) is a potent cell proliferating estrogen whereas 2-methoxyestradiol (2-ME₂) is a potent inhibitor of cell proliferation through the activation of apoptosis. 2-Hydroxyestradiol (2-HE₂) also causes increased cell proliferation but it is not as potent as 4-HE₂ and it is *O*-methylated rapidly to 2-ME₂. 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-HE₂/2-ME₂ ratio. In this study, an analytical method was developed to measure the concentration levels of E₂, 2-ME₂, 2-HE₂ and 4-HE₂ 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 (1×10^6 cells/flask) of MCF-7 and MCF-12A cells were provided with 11 ml medium containing $E_2/2\text{-HE}_2/4\text{-HE}_2/2\text{-ME}_2$ (10^{-6} M) and medium containing $E_2/2\text{-HE}_2/4\text{-HE}_2/2\text{-ME}_2$ (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 katesjoolmetaboliëte van estradiol karsinogene veroorsaak. 4-Hidroksie estradiol (4-HE₂) is 'n sterk selprolifererende estrogeen terwyl 2-metoksie estradiol (2-ME₂) 'n sterk inhibitor is van selproliferasie deur die aktivering van apoptose. 2-Hidroksie estradiol (2-HE₂) veroorsaak ook verhoogde selproliferasie, maar tot 'n mindere mate as 4-HE₂ en dit word vinnig ge-O-metileer na 2-ME₂. Die katesjoolmetaboliëte 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 karsinogene. 'n Vername verhouding om ingedagte te hou as 'n biomerker vir borskankerrisiko is die 4-HE₂/2-ME₂ verhouding. In hierdie studie is 'n analitiese metode ontwikkel om die konsentrasievlakke van E₂, 2-ME₂, 2-HE₂, en 4-HE₂ in selkultuurmedium te meet. Die metode maak gebruik van gaschromatografie-massaspektrometrie (GC-MS) analise omdat die verwagte fisiologiese konsentrasies van hierdie metaboliëte 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 diëtleter en derivatisering met trimetielsilielimidazool (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 metaboliëte 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 (1×10^6 selle/fles) van MCF-7 en MCF-12A selle was voorsien van 11 ml medium wat $E_2/2\text{-HE}_2/4\text{-HE}_2/2\text{-ME}_2$ (10^{-6} M) en medium wat $E_2/2\text{-HE}_2/4\text{-HE}_2/2\text{-ME}_2$ (10^{-8} M) bevat. Vir elke metaboliëte 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 1 uur 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 metaboliëte 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 verkrygte resultate.

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Abbreviations

•OH	hydroxyl radical
16 α -HE ₁	16 α -hydroxyestrone
16 α -HE ₂	16 α -hydroxyestradiol
17 β -HSD	17 β -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
5 β -C	5 β -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 <i>O</i> -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- <i>p</i> -dioxin
TDLU	terminal ductal lobular units
TIC	total ion chromatogram
TMS	trimethylsilyl
TMSI	trimethylsilylimidazole
WCOT	wall coated open tubular