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Chromatographic Studies Of Some Hormone And Metabolic Modulators Used For Doping In Sports

Ruth Marie Speidel
Eastern Kentucky University

CHROMATOGRAPHIC STUDIES OF SOME HORMONE AND METABOLIC MODULATORS USED FOR DOPING IN SPORTS

BY

RUTH SPEIDEL

THESIS APPROVED:

Karim Abdelhay
_____
Chair, Advisory Committee

jamie fredericks
_____
Member, Advisory Committee

Jerome Nagy
_____
Member, Advisory Committee

Cynthia J. K Tsou
_____
Member, Advisory Committee

Ruy K. Bazzetto
_____
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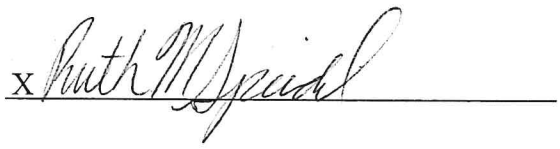

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RUTH SPEIDEL

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ABSTRACT

The World Anti-Doping Agency (WADA) is an international agency that monitors the use of Performance Enhancing Drugs (PEDs) in sports; their mission is “to lead a collaborative worldwide movement for doping-free sport” (Who We Are, 2022). For this mission, the WADA needs quick, sensitive, reproducible, and accurate methods for analyzing athletes’ biological fluids for possible PEDs. This research focuses on developing and validating simple, sensitive, rapid, and reliable gas chromatography-mass spectrometry (GC-MS) methods for the analysis of two hormone and metabolic modulators, toremifene and trimetazidine. Previously, there has been limited GC-MS methods used to analyze toremifene and trimetazidine. Quantification of these two PEDs was based on the peak area instrument response. The performance of the proposed methods was validated for linearity, accuracy, precision, sensitivity, and limits of detection (LOD) and quantification (LOQ) according to the International Council on Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines. Furthermore, the developed method was applied for the determination of trimetazidine in urine using liquid-liquid extraction with a 37% extraction efficiency.

TABLE OF CONTENTS

List of Abbreviations

AAF .....Adverse Analytical Finding

EI .....Electron Ionization

E_r.....Error

GC.....Gas Chromatography

GC-MS-NPD .....Gas Chromatography-Mass Spectrometry-Nitrogen Phosphate Detector

HETP .....Height Equivalent to one Theoretical Plate

HPLC .....High Performance Liquid Chromatography

HPLC-DAD.....High Performance Liquid Chromatography-Photo Diode Array Detector

ICH.....International Council on Harmonization of Technical Requirements for Pharmaceuticals for Human Use

LC/ESI-MS.....Liquid Chromatography-Electron Spray Ionization Mass Spectrometry

LC-MS .....Liquid Chromatography- Mass Spectrometry

LOD.....Limit of Detection

LOQ ..... Limit of Quantitation

MS .....Mass Spectrometry

m/z ..... mass-to-charge ratio

PEDs ..... Performance Enhancing Drugs

r^2..... Correlation Coefficient

RSD ..... Relative Standard Deviation

S_a .....Standard Deviation of the Intercept

S_b .....Standard Deviation of the Slope

$S_{y/x}$ ..... Standard Deviation of the Residuals

SERM .....Selective Estrogen Receptor Modulator

SIM ..... Single Ion Monitoring

TIC ..... Total Ion Chromatogram

WADA .... World Anti-Doping Agency

List of Equations

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

I. Introduction

World Anti-Doping Agency (WADA)

The World Anti-Doping Agency (WADA) is the leading international organization for the collaboration for doping free sports (Who We Are, 2022). In order to maintain and enforce doping free sports, the WADA needs quick and reliable methods to determine the presence of Performance Enhancing Drugs (PEDs). Doping began as early as the third century, through various remedies to enhance athletic performance; despite its early appearance, the ban of doping for athletes internationally did not start until 1928 (Mullar, 2010). Testing and disqualifications for the use of PEDs were not possible until the Olympics in 1968 (Mullar, 2010).

The WADA separates the PEDs into different classification according to their effects on the body. The PEDs are also separated based on if they are banned at all times or just during competition (Prohibited List, 2020). The PEDs that are prohibited at all times typically have longer lasting effects, such as anabolic agents and hormone and metabolic modulators. Subsequentially, the PEDs that are banned only during competition have shorter effects such as stimulants and narcotics.

Anti-estrogen medications have been on the WADA's Prohibited List since the list first came out in 2004 (Prohibited List, 2004). Originally only four items were included in that list: aromatase inhibitors, clomiphene, cyclofenil, and tamoxifen. The last three items on that list are under another classification of PEDs now known as Selective Estrogen Receptor Modulators (SERMs) (Prohibited List, 2020). Since then, the list has grown, and the SERMs have been classified as a type of hormone and metabolic modulator. They are joined in this class by aromatase inhibitors, agents

preventing activin receptor activation, and metabolic modulators, such as insulin and trimetazidine (Prohibited List, 2020).

SERMs have become a common alternative to anabolic steroids due to its similar effect of increasing testosterone in men with less severe side effects (Hackney, 2018). In fact, every year the WADA releases reports of all the testing and the adverse analytical findings (AAF). Since 2017, hormone and metabolic modulators have accounted for approximately 8% of the AAF. The 17% AAF spike in 2016 seen in **Table 1**, was caused by a new PED, meldonium, being added to the hormone and metabolic modulator list (Anti-Doping Testing Figures. 2015-2020).

Table 1: WADA Recorded Number of Hormone and Metabolic Modulators AAF

Year	Number of Adverse Analytical Findings	Number of Hormone and Metabolic Modulator AAF	Percent of AAF due to Hormone and Metabolic Modulators
2015	3432	152	4%
2016	4234	721	17%
2017	4076	321	8%
2018	4117	350	9%
2019	4180	362	9%
2020	1513	127	8%

Data from Anti-Doping Testing Figures 2015-2020

Trimetazidine accounts for approximately 2% of the hormone and metabolic modulators AAF each year or approximately 0.16% of the total AAF (Anti-Doping Testing Figures. 2015-2020). Most recently in the 2022 Winter Olympics, a Russian Figure Skater had a positive drug test for the metabolic modulator trimetazidine. The WADA has not yet made a decision if this would revoke the gold medal Russia won for women's figure skating (Ritchia, 2022). This PED is one of the drugs of focus for this research and will be discussed later.

The other drug of focus in this research is toremifene. In the last 5 years of released records (2015-2020) of the WADA, toremifene has not been recorded as an AAF (Anti-Doping Testing Figures. 2015-2020). It is unclear why toremifene has not been detected by the WADA. While it is possible that it is a result of no athletes using toremifene, it is also possible that the WADA is not testing for it or cannot test for it.

Aims and Objectives

Currently, there is little research in analyzing the hormone and metabolic modulators, toremifene and trimetazidine, using gas chromatography-mass spectroscopy (GC-MS). This research contributes to bridge the gap in instrumental techniques in analyzing toremifene and trimetazidine both in bulk and in biological fluid. This research contributes the WADA with the development of simple, sensitive, economic, and fast analytical methods in order to continue the movement in doping free sports.

The objectives of this research are summarized in the following four specific aims:

1. Developing sensitive, accurate and reproducible analytical methods that can detect and determine some PEDs in bulk and in biological fluids.

2. Study how different mass spectrometric scanning modes (Total Ion Chromatogram “TIC” and Single Ion Monitoring “SIM”) affect the sensitivity and linearity of the proposed analytical methods.

3. Study different types of calibration e.g., external calibration and internal-standard calibration in the analysis of different PEDs.

4. Validation of the proposed analytical methods according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines.

Analytical Techniques

Gas Chromatography

Chromatography is "the separation of components of a mixture by a series of equilibrium operations" (Grob, 2004). These equilibrium operations are occurring with the analyte between the stationary and mobile phases. The rate at which a component travels through the chromatography column is the sum of transportation rate by the mobile phase and the retention of the component in the stationary phase (Engewald, 2014).

Plate Theory and the Van Deemter Equation

The column used in chromatography can be separated into theoretical plates. This divides the separation process into discrete steps where at each plate there is an equilibrium of the analyte between the mobile and stationary phases (Engewald, 2014). The number of plates is defined by the **Equation 1**, where N is the number of theoretical plates, t_r is the retention time, and w is the width of the peak at the base.

$$N = 16(^{t_r}/_{w})^2 \qquad (1)$$

Equation 1: Number of Theoretical Plates

$$HETP = L/N \qquad (2)$$

Equation 2: Height Equivalent to One Theoretical Plate

The height equivalent to one theoretical plate (HETP), **Equation 2**, is the length of the column divided by N. A larger number of theoretical plates and smaller plate height indicates a more efficient column and higher resolution (Engewald, 2014).

The HETP can also be defined by the Van Deemter Equation, shown in **Equation 3**, where A is the eddy diffusion term, B is the longitudinal diffusion term, C is the mass transfer term, and μ is the flow rate of the mobile phase.

$$HETP = A + \frac{B}{\mu} + C\mu \qquad (3)$$

Equation 3: Van Deemter Equation

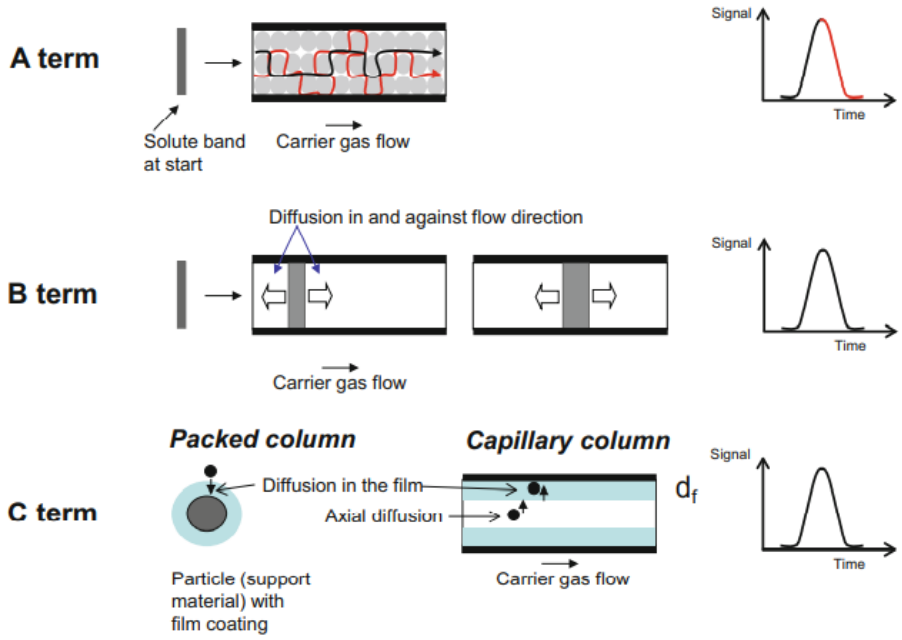

Figure 1: Visual Representation of the Van Deemter Variables

Source: Engewald, W. Dettmer-Wilde, K. Theory of Gas Chromatography in *Practical Gas Chromatography*, Springer-Verlag Berlin Heidelberg 2014

The Van Deemter Equation explains the broadening of peaks in relation to the flow rate of the mobile phase (Engewald, 2014). Eddy diffusion (A) refers to the different paths a compound can take through a packed column. The column used in

this research is not a packed column, so the A term can be ignored. The longitudinal diffusion (B) is caused by the differences in concentration as the analyte band moves through the column. As with normal diffusion, analyte flows from areas of high concentration to areas of low concentration. The diffusion causes broadening of the analyte band as it moves down the column, as seen in **Figure 1**. The mass transfer between the stationary phase (C_s) and mobile phase (C_m) are determined by the diffusion of the analyte in the stationary and mobile phases, meaning that some analyte will be moving with the mobile phase and some analyte will be stopped by the stationary phase.

Advantages and Limitations of Gas Chromatography

Gas chromatography has high sensitivity, separation ability, and resolution, but it can only be applied to volatile and thermally stable compounds. In comparison to liquid chromatography, such as high-pressure liquid chromatography (HPLC), GC has a longer column length allowing the separation of complex mixtures with higher resolution. The better resolution can also be linked back to the Van Deemter equation. Many liquid chromatography columns are packed, meaning the Eddy Diffusion variable (A in **Equation 3**) is significant, unlike in GC. This leads to larger HETP and lower resolutions. HPLC is also more expensive than GC since it needs a larger number of organic solvents. The disadvantage of GC compared to HPLC is that HPLC is more selective than GC due to being able to easily change the mobile phase. These advantages and limitations are summarized in **Table 2**.

Table 2: Advantages and Limitations of Gas Chromatography and High-Pressure Liquid Chromatography

Gas Chromatography	High Pressure Liquid Chromatography
Advantages	
• Higher Resolution and Separation • Cheaper	• Higher Selectivity
Limitations	
• Can only analyze volatile and thermally stable compounds	• More Expensive

Resolution

Resolution refers to the degree of separation between two peaks and is defined by **Equation 4**, where R_s is the resolution, t_r is the retention time, and w is the width of the peak at its base (Engewald, 2014).

$$R_S = \frac{t_{r(2)} - t_{r(1)}}{(w_2 + w_1)/2} \qquad (4)$$

Equation 4: Resolution

This equation works for peaks of similar heights and without tailing. The peaks are considered baseline separated at R_s= 1.5. The resolution can be increased by narrowing the peaks with better column efficiency or increasing the distance between peaks.

Mass Spectrometry

Mass spectrometry involves the generation of ions, the separation of these ions by their mass-to-charge ratio (m/z), and their detection in respect to their m/z and abundance (Gross, 2006). In this research, the ionization involves bombarding a neutral analyte with high energy electrons to form a radical ion:

$$M + e^- \rightarrow M^{+\cdot} + 2e^-$$

This mode of ionization is referred to as electron ionization (EI). The radical is most likely to occur at a lone pair of electrons. The EI source is a type of hard ionization, meaning that enough energy is transferred to the compound that it fragments at the weaker bonds.

The separation of the ions by their m/z values is done by a mass analyzer. Typically, with chromatography a quadrupole is used as the mass analyzer. The quadrupole has four parallel metal rods to which constant voltage and radio-frequency oscillating voltage is applied (Harris, 2010). These voltages direct the ions into helical trajectories, so that only ions of a specific m/z value can reach the detector. The voltages are also rapidly changing so ions of different m/z can reach the detector (Harris, 2010).

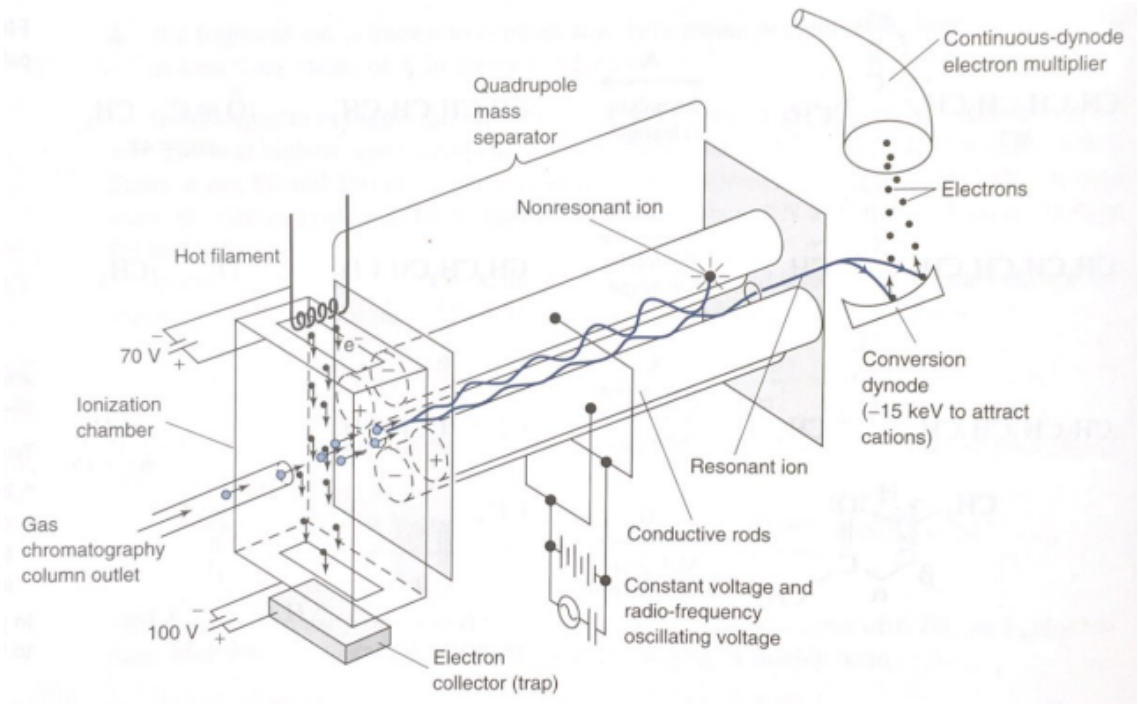

Figure 2: EI and Quadrupole Mass Spec
Source: Harris, D.C. Mass Spectrometry in *Quantitative Chemical Analysis*, Macmillan Education 2016

The GC-MS can be run in two different modes: Total Ion Chromatogram (TIC) and Single Ion Monitoring (SIM). The TIC measures a range of ions and plots all of the detected m/z ions as a function of intensity. The SIM measures only the selected m/z ratio(s) (Gross, 2006).

Calibration

In this research, two different calibration methods were used: external standard calibration and internal standard calibration. External standard calibration uses samples of known but varying concentrations; these are plotted against the instrumental response (peak areas from the GC) to form a calibration curve(Harris, 2010). An internal standard is used when there is low instrument precision or to correct for interferences due to biological sample matrix. To construct a calibration curve with an internal standard, the analyte will have varying concentration; however, the internal standard will have a consistent concentration in each standard. The ratio of concentration of the analyte and internal standard is plotted against the ratio of instrument responses. An internal standard should have a distinct instrument response from the analyte, be chemically similar to the analyte, and should not react with the analyte or the matrix (Harris, 2010).

In this research two different internal standards were used. Tamoxifen was selected as an internal standard for toremifene. Toremifene is the chlorinated form of tamoxifen (Taras, 2000); as such the two have similar chemical structures and properties. These structures are depicted in **Figure 3**.

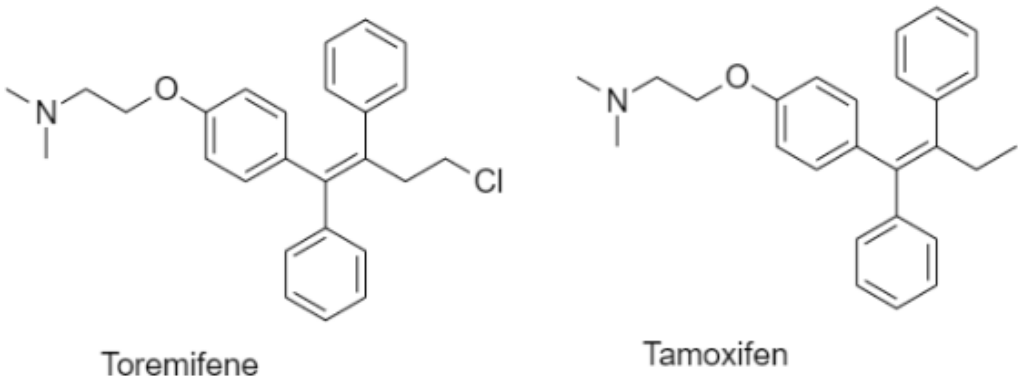

Figure 3: Chemical Structures of Toremifene and Tamoxifen

Propranolol was selected as an internal standard for trimetazidine. The selection of propranolol as an internal standard was based off of an article by *Mistri et al*. (2003). Another possible internal standard for trimetazidine is lidocaine (Jiao, 2007).

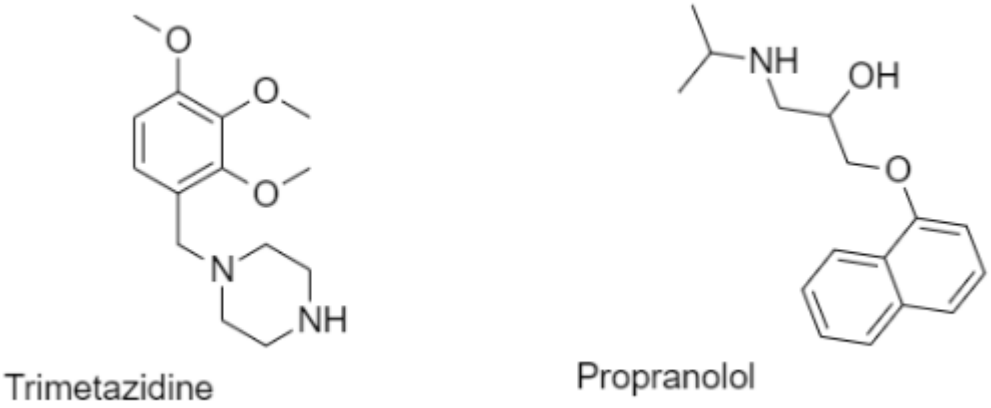

Figure 4: Chemical Structures of Trimetazidine and Propranolol

Validation of the Developed Analytical Methods

The purpose of validating an analytical method is to show that it is suitable for its intended purpose (ICH, 2005). The International Conference on Harmonisation of

Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) collects and defines the terms used for validating methods (ICH, 2005).

Linearity is the ability of the procedure to produce results that are directly proportional to the concentration of the analyte (ICH, 2005). The linearity is determined by analyzing different samples with different concentration of analyte. The concentration of the samples is plotted on the x-axis, while the instrument response (peak area) of the samples is plotted on the y-axis. The best straight line is drawn through experimental data points using the method of least squares (Zou, 2003), where the linear equation is y=bx+a (b is the slope and a is the y-intercept). The slope (b) of the line of best fit measures the sensitivity of the analytical method (Zou, 2003). The strength of the linear relationship can be defined by the proportion of variance (r^2) (Zou, 2003). The closer the r^2 value is to 1, the more linear of a relationship there is between the concentration and the instrument response.

Accuracy is the closeness of agreement between the measured value and the accepter or true value (ICH, 2005). The accuracy was determined using the external calibration; three different analyte concentrations were analyzed five times in sequence on three different days. The concentrations were extrapolated from the external standard calibration curve. The accuracy can be determined by the percent error equation, **Equation 5** (Harris, 2010). The smaller the $\%E_r$ is, the more in agreement the measured (experimental) value is to the labelled (true) value, meaning it is more accurate. To be in agreement with the ICH guidelines the $\%E_r$ needs to be less than 5% in order to be considered accurate.

$$\%E_r = \frac{|experimental - true|}{true} * 100\% \qquad (5)$$

Equation 5: Percent Error Equation

Precision is the degree of scatter or closeness of agreement between a series of measurements (ICH, 2005). In other words, how repeatable are the results. Intra-day or repeatability is the precision of one batch of samples on one day. Three different analyte concentrations were analyzed five times in sequence. Inter-day or intermediate precision is the precision of the method spanning across different days. Three different analyte concentrations were analyzed five times in sequence on three different days. Precision can be calculated by the percent relative standard deviation (%RSD), which is defined by **Equation 6** where $\bar{x}$ is the average and SD is the standard deviation (Harris, 2010). The smaller the %RSD is, the closer in agreement the data points are, and the better the precision. To be in agreement with the ICH guidelines the %RSD needs to be less than 2% in order to be precise.

$$\%RSD = \frac{\bar{x}}{SD} * 100\% \qquad (6)$$

Equation 6: Percent Relative Standard Deviation

The robustness of a procedure is its ability to remain unaffected by small changes to method parameters (ICH, 2005). The robustness can be analyzed by intentionally varying the instrumental method. For gas chromatography typical variations are done to the inlet and oven temperatures (ICH, 2005).

The limit of detection (LOD) is the lowest amount of analyte that can be detected and is defined by **Equation 7**, where S_a is the standard deviation of the intercept and b is the slope. The limit of quantitation (LOQ) is the lowest amount of analyte that can be quantified with accuracy and precision and is defined by **Equation 8** (ICH, 2005). Both **Equation 7 and 8** use the standard deviation of the intercept; this is the amount of error or uncertainty of the intercept of the line of best fit(Stone, 2022). The LOQ is also three times the LOD.

$$LOD = \frac{3.3S_a}{b} \qquad (7)$$

Equation 7: Limit of Detection Equation

$$LOQ = \frac{10S_a}{b} = 3 * LOD \qquad (8)$$

Equation 8: Limit of Quantitation Equation

Both the LOD and the LOQ are calculated based on the calibration curve; however, it can also be determined based on the signal-to-noise ratio. A larger signal-to-noise ratio indicates a higher LOD and LOQ. For this reason, tall narrow peaks are preferred, which can be optimized by having smaller HETP.

II. Experimental Parameters

Instrumentation

The GC-MS analysis was conducted using an Agilent Technologies 7890B GC and 5977B MSD systems. The GC was operated in splitless mode with helium carrier gas flow rate of 0.9 mL/min and a column head pressure of 10 psi. The mass spectrometer was operated in the electron impact mode using and ionization voltage of 70 eV and source temperature of 230°C. The gas chromatograph injector was maintained at 250°C and the auxiliary heater at 280°C. The chromatographic separations and collection of retention data were carried out on a 30-meter 0.25-micron stationary phase HP-5ms (5%-phenyl)-methlpolysiloxane.

Drugs and Chemicals

Toremifene was purchased from Alfa Aesar (Ward Hill, MA). Tamoxifen was purchased from Cayman Chemicals (Ann Arbor, MI). Trimetazidine Dihydrochloride was purchased from TCI America (Portland, OR). Propranolol was purchased from Cayman Chemicals (Ann Arbor, MI). HPLC grade methanol was purchased from Fisher (Waltham, MA). Chloroform was purchased from EKU college of STEM chemical storage facility. Sodium Hydroxide 22% solution was purchased from Alfa Aesar (Ward Hill, MA). The artificial urine was purchased from Ward's Natural Science (Rochester, NY).

III. Analysis of Toremifene

Toremifene (brand name: Fareston) is a selective estrogen receptor modulator (SERM) that is prohibited to athletes (Prohibited List, 2020). Medically, toremifene and other SERMs are used to treat estrogen binding breast cancer, osteoporosis, and some cardiovascular diseases in postmenopausal women (Taras, 2000). Toremifene is usually administered orally with peak plasma concentration within 2-4 hours and 99% bound to plasma proteins (Wiseman, 1997). Toremifene does get metabolized by the liver; the metabolic pathway of toremifene is depicted in **Figure 5** (Taras, 2000). The metabolites resulting from the N-demethylation and oxidations have been detected in plasma along with the parent drug, toremifene (Taras, 2000).

Toremifene and its metabolites have an elimination half-life (50% of the drug is excreted) of 5 days, mostly in the feces (Taras, 2000). Like other SERMs, toremifene is used as a PEDS because it in an antiestrogen and increases the levels of testosterone in men (Hackney, 2018).

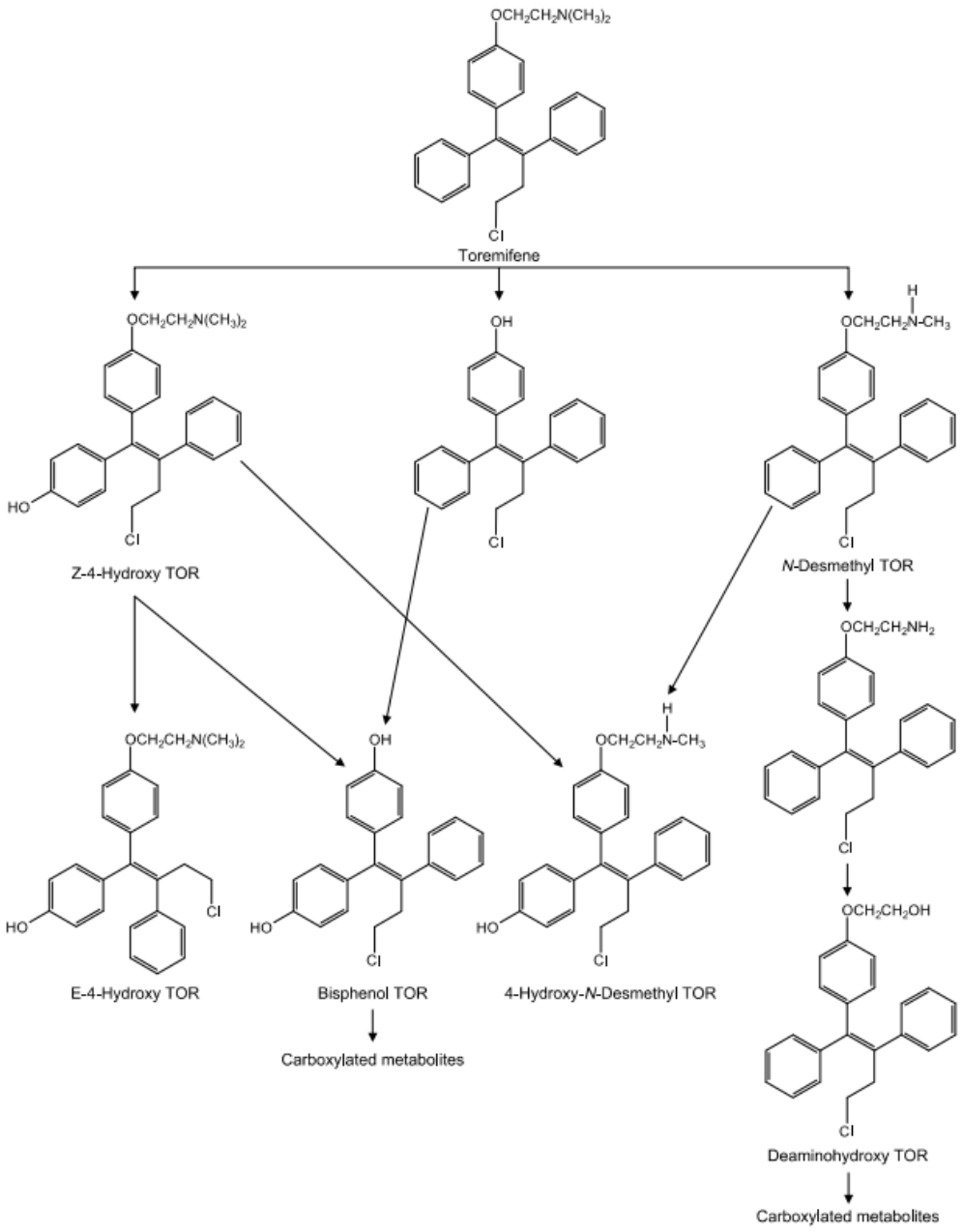

Figure 5: Metabolic Pathway of Toremifene
Source: Taras, T.L.; Wurz, G.T.; Linares, G.R.; DeGregorio, M.W. Clinical Pharmacokinetics of Toremifene. *Clin Pharmacokinet* 2000, 39(5): page 332

Currently, there is little reported research on the analysis of Toremifene using GC-MS. Previously, methods for analyzing Toremifene involved the use of High-

Performance Liquid Chromatography (HPLC) (Webster, 1991) and Liquid Chromatography-Mass Spectrometry (LC-MS) (Martinsen, 1996; Watanabe, 1989).

The reported HPLC method used reversed-phase C_{18} column with a pH=6.4 65:35 acetonitrile: 100mM ammonium acetate mobile phase. This method had a ten-minute run time and a linear range of 0.1-10 $\mu g*mL^{-1}$ (Webster, 1991). The authors of this method did not report the Limit of Detection or Quantitation values but did report a level of precision ranging from 2-18% variance in their intra-assay testing and 2-16% in their inter-assay testing (Webster, 1991).

One of the LC/MS methods used a cyano column with a pH=8 70:30 methanol: 0.1 M ammonium acetate mobile phase. The mass spectrometer used an atmospheric pressure ionization and double focusing mass analyzer. This method had a twenty-minute run time. This method was not to quantify the amount of toremifene, but rather test the presence of toremifene and its metabolites after five daily doses of 480 mg were administered to breast cancer patients, so no analytical parameters were given, but the authors were able to separate and detect toremifene in the presence of two of its metabolites, N-desmethyltoremifene and 4-hydroxy-N-desmethyltoremifene (Watanabe, 1989).

Another LC/MS method used a C_8 column with a pH=6.5 65:35 acetonitrile: 0.1M ammonium acetate mobile phase. The mass spectrometer used a thermospray ionization source with a quadrupole mass analyzer and SIM set to 406 m/z. This method had a 25-minute run time and linear concentration range of 0.01-10 $\mu g*mL^{-1}$.

Similar to the previous method, the authors did not test the analytical parameters; they just tested the ability to detect toremifene (Martinsen, 1996).

Experimental

External Calibration

Toremifene stock solution was prepared by dissolving the powder in methanol. The calibration solutions were prepared by dilution of aliquots of the toremifene stock solution with methanol to reach the concentration range 0.025-0.1 $mg*mL^{-1}$. The solutions were chromatographed using the GC-MS with both TIC and SIM with m/z=58. The peak areas of toremifene were plotted against the concentration to obtain the calibration curve. Microsoft Excel was used to obtain linear regression parameters, such as the proportion of variance and standard deviations of the slope, intercept, and residuals.

Internal Standard Calibration

Tamoxifen was selected as the internal standard. Tamoxifen stock solution was prepared by dissolving standard powder in methanol. Toremifene calibration solutions were spiked with 0.1 $mg*mL^{-1}$ of tamoxifen. The solutions were chromatographed using the GC-MS. The ratio of the instrument response of toremifene/tamoxifen were plotted against the ratio of concentration toremifene/tamoxifen to obtain the calibration curve.

Results and Discussions

Method Development

There is little published information on the analysis of toremifene using GC-MS. The first method that had a successful elution of toremifene was helium carrier gas flow rate of 0.9 $mL.min^{-1}$ and a column head pressure of 10 psi. The inlet temperature was 250°C and the auxiliary heater at 280°C; these instrument parameters were kept constant while the temperature program was adjusted as seen in **Table 3** to lower retention time.

Table 3: Temperature Programs and Retention Times for the Method Development of Toremifene using TIC at 0.1 $mg*mL^{-1}$

Method	Temperature Program			Retention Time of Toremifene
	Ramp ($°C.min^{-1}$)	Temperature (°C)	Hold (min)	
TOR-M1		110	1	24.9 minutes
	7	180	3	
	15	280	5	
	30	340	3	
TOR-M2		110	1	20.8 minutes
	10	180	3	
	20	280	5	
	40	340	5	
TOR-M3		110	1	16.8 minutes
	20	230	5	
	20	340		
TOR-M4		110	1	15.4 minutes
	20	230	5	
	30	340	2	

These retention times were overlaid in **Figure 6**. Using **Equation 1 and 2**, the number of theoretical plates was calculated for each of the methods and is presented in **Table 4**. As the temperature program was optimized, the peaks got more narrow

and taller; this also coincided with the shorter theoretical plate heights. The shorter plate height indicated a higher resolution and more efficient separation. Because of this, the final method, TOR-M4, was used for the rest of the analysis of toremifene.

Table 4: Theoretical Plates and HETP for Toremifene

Method	Theoretical Plates	HETP (meters)
TOR-M1	661000	$4.5*10^{-5}$
TOR-M2	410000	$7.3*10^{-5}$
TOR-M3	1750000	$1.7*10^{-5}$
TOR-M4	2090000	$1.4*10^{-5}$

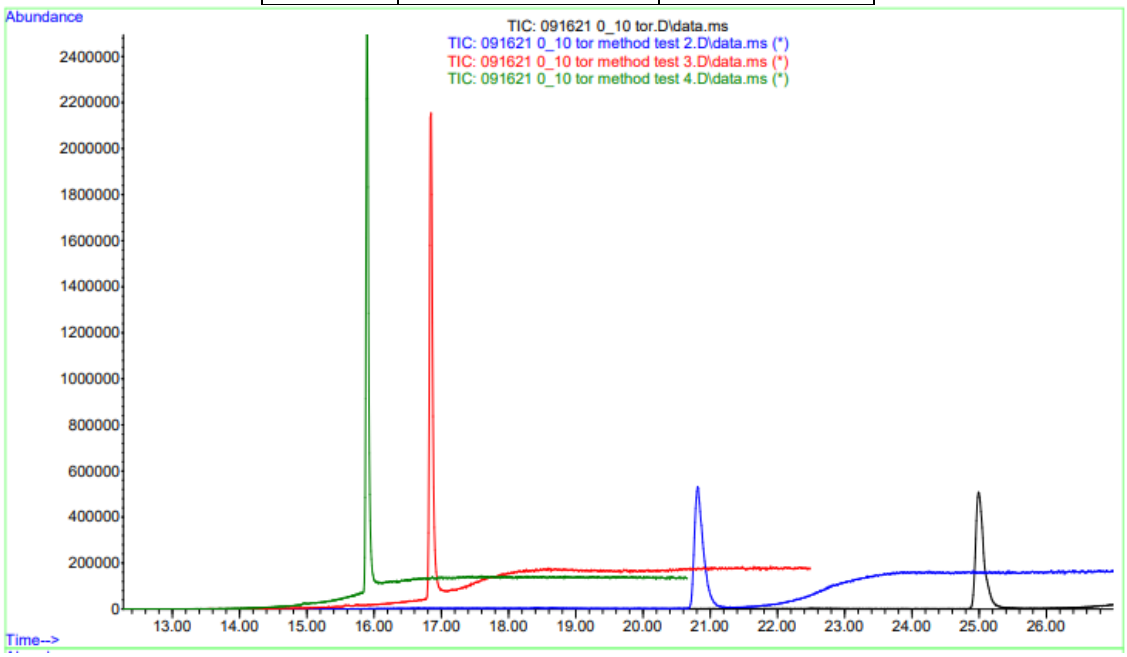

Figure 6: Toremifene Method Development Overlay using TIC at 0.1 mg*mL^{-1}
TOR-M1 (Black), TOR-M2 (Blue), TOR-M3 (Red), TOR-M4 (Green)

The above data was all collected with using TIC. For the SIM that will be mentioned in the next section, a m/z of 58 Da was chosen. This fragment was from the amine group $(CH_3)_2NCH_2^{\cdot+}$ on the parent drug. As seen on the metabolic pathway, **Figure 5**, most of the metabolites are demethylated at this amine group. These metabolites will not have a mass fragment at m/z of 58 Da. Using SIM with a m/z of 58 Da, may help distinguish the parent drug, toremifene, from its metabolites.

Analytical Performance

The linearity for the procedure was analyzed by using different concentrations of toremifene. **Table 5** presents the statistical parameters of the linear regression including the concentration range, proportion of variance (r^2), LOD, and LOQ. Other linear regression analytical parameters are documented in **Appendix A**. Since the proportion of variance was higher than 0.99, there was a strong linear correlation between the concentration of toremifene and the instrument response.

Table 5: External Standard of Toremifene Linear Regression Analytical Parameters

Parameter	Toremifene TIC	Toremifene SIM m/z= 58
Concentration range ($mg*mL^{-1}$)	0.0250-0.0750	0.0250-0.10
Proportion of variance (r^2)	0.993	0.991
Limit of Detection LOD ($mg*mL^{-1}$)	0.0149	0.0153
Limit of Quantitation LOQ ($mg*mL^{-1}$)	0.0448	0.0463

The LOD and LOQ were similar for the TIC and SIM for the external standard calibration of toremifene. Due to the steeper slope demonstrated in **Figure 7**, the SIM showed a higher level of sensitivity compared to TIC, meaning it was better at detecting change in concentrations than the TIC.

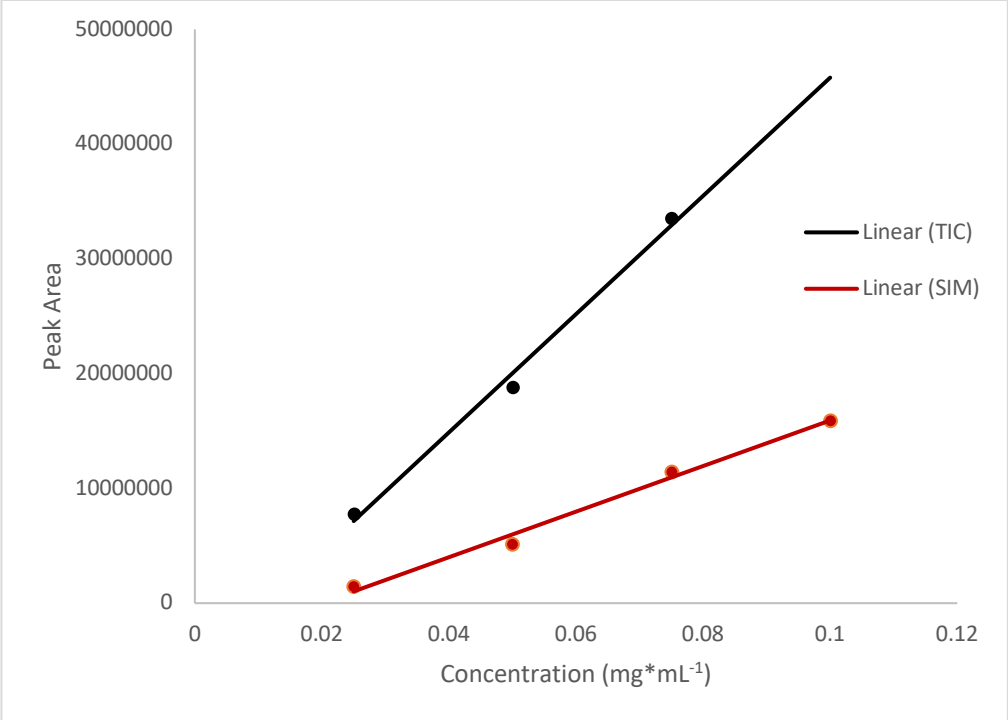

Figure 7: External Standard TIC and SIM Linear Regression for Toremifene

Internal Standard

Tamoxifen was chosen as an internal standard for toremifene. Using the same GC method as mentioned previously, tamoxifen eluted at approximately 14 minutes, as seen in **Figure 8**. Furthermore, tamoxifen and toremifene shared a similar mass-to-charge fragment at m/z=58 Da. With the similar but distinguishable instrument response, tamoxifen was suitable as an internal standard for toremifene.

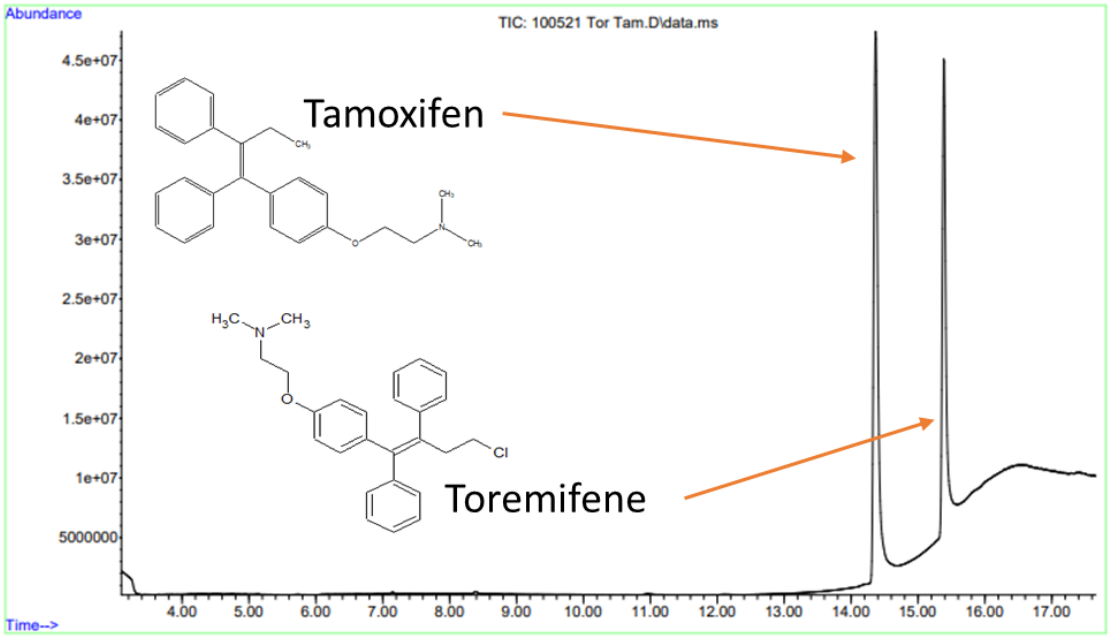

Figure 8: Gas Chromatogram of Tamoxifen and Toremifene

Resolution measures how well two peaks can be distinguished on the chromatogram, and it is defined by **Equation 4**. There was sufficient baseline resolution between the tamoxifen (retention time 14.4) and the toremifene (retention time 15.4) peaks with a calculated baseline resolution of 15.9.

As before, the linearity was analyzed by using different concentrations of toremifene spiked with 0.1 $mg*mL^{-1}$ of tamoxifen. **Table 6** presents the same analytical parameters as **Table 5**. The LOD and LOQ in **Table 6** were calculated as a ratio of the concentration of toremifene and tamoxifen. Since the concentration of tamoxifen was 0.1 $mg*mL^{-1}$ for all samples, the LOD for toremifene was 0.0256 $mg*mL^{-1}$ and 0.0350 $mg*mL^{-1}$ for TIC and SIM respectively. The LOQ for toremifene was 0.0767 $mg*mL^{-1}$ and 0.105 $mg*mL^{-1}$ for TIC and SIM respectively. These values are also recorded in **Table 6**.

Table 6: Internal Standard Linear Regression Analytical Parameters for Toremifene

Parameter	Toremifene/Tamoxifen TIC	Toremifene/Tamoxifen SIM m/z=58
Concentration range ($mg*mL^{-1}$)	0.0250-0.150	0.0250-0.150
Correlation coefficient (r^2)	0.987	0.976
Limit of detection (LOD) ($mg*mL^{-1}$)	0.256	0.350
Limit of quantitation (LOQ) ($mg*mL^{-1}$)	0.767	1.05
	Toremifene TIC	Toremifene SIM
Limit of detection (LOD) ($mg*mL^{-1}$)	0.0256	0.0350
Limit of quantitation (LOQ) ($mg*mL^{-1}$)	0.0767	0.105

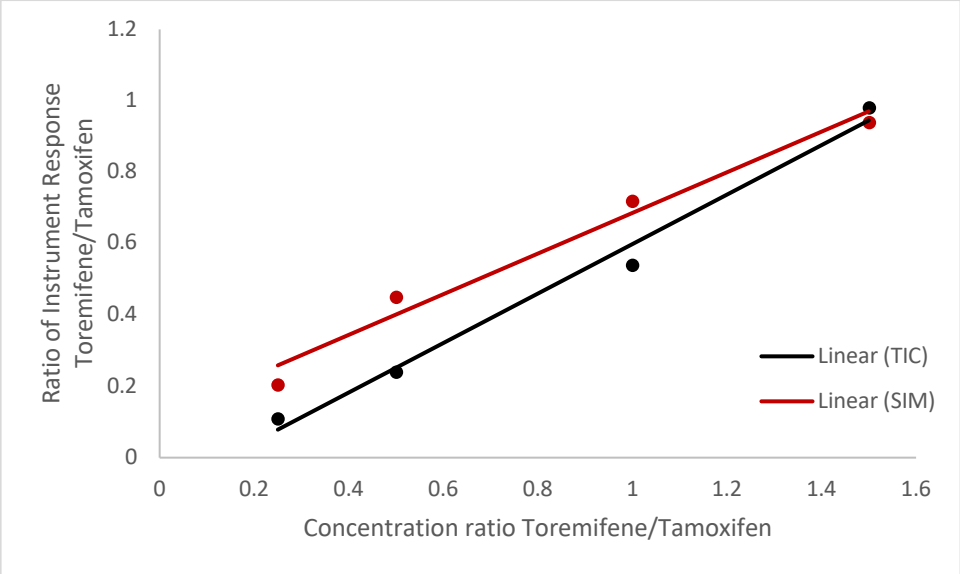

Figure 9: Internal Standard Toremifene/Tamoxifen TIC and SIM Linear Regression

In **Figure 9**, the TIC showed a steeper slope and therefore was a more sensitive method compared to the SIM with an internal standard. With the internal standard, the LOD and LOQ were about double of that with just the external standard. Also, there was less of a linear relationship between the concentration and the instrument response. A reason for this is that an internal standard corrects for poor instrument

precision. If there was already sufficient instrument precision, then an internal standard would not improve the analytical parameters.

Method Validation

Table 7: Toremifene TIC Precision and Accuracy

Labeled Concentration ($mg*mL^{-1}$)	Found Concentration ± Standard Deviation ($mg*mL^{-1}$)	%RSD	$\%E_r$
Intra-day (Repeatability)			
0.05	0.044 ± 0.003	8.28%	12.6%
0.075	0.072 ± 0.001	1.32%	3.19%
0.1	0.094 ± 0.0008	0.83%	6.09%
Inter-day (Intermediate Precision)			
0.05	0.037±0.005	14.2%	26.4%
0.075	0.075 ± 0.002	2.05%	0.13%
0.1	0.099 ±0.003	0.32%	0.71%

n=5

In order to test the method repeatability, three different concentrations of Toremifene were tested with both TIC (**Table 7**) and SIM (**Table 8**) without the presence of an internal standard a total of five times in sequence. The intermediate precision was also tested by running the same concentration on three different days.

Table 8: Toremifene SIM Precision and Accuracy

Labeled Concentration ($mg*mL^{-1}$)	Found Concentration ± Standard Deviation ($mg*mL^{-1}$)	%RSD	$\%E_r$
Intra-day (Repeatability)			
0.05	0.051 ± 0.002	4.57%	1.31%
0.075	0.073 ± 0.001	0.25%	2.58%
0.1	0.095 ± 0.0005	0.55%	5.14%
Inter-day (Intermediate Precision)			
0.05	0.049±0.002	4.84%	2.93%
0.075	0.076 ± 0.01	0.45%	1.89%
0.1	0.099 ±0.01	0.82%	0.71%

n=5

The $\%E_r$ values were found to be less than 5% which indicates that the proposed method was accurate. The %RSD values were found to be less than 2% which complies with the ICH guidelines and indicated that the proposed method was precise. At 0.05 $mg*mL^{-1}$, the accuracy and precision was not included in the acceptable range for accuracy and precision. This was most likely due to how close this was to the LOQ reported in **Table 5**. The LOQ is 0.046 $mg*mL^{-1}$, so the closer the analyte was to that concentration the less accurate and precise it was.

The robustness (**Appendix B**) of the method was analyzed by intentionally varying the temperature program, inlet temperature, and auxiliary temperature by 2°C. These changes visually did not significantly change the instrument response of toremifene.

IV. Analysis of Trimetazidine

Trimetazidine is a metabolic modulator that is prohibited at all times for athletes (Prohibited List, 2020). Medically, trimetazidine is used to treat coronary diseases such as angina pectoris. It is administered orally and only weakly binds to plasma proteins. Trimetazidine metabolizes as seen in **Figure 10**; while there are several metabolites, trimetazidine only biotransforms to a low extent (Dezsi, 2016). Trimetazidine has an excretion half-life of approximately 6 hours and mostly through the urine (Dezsi, 2016). For doping, trimetazidine optimizes cardiac energy metabolism; this increases the level of endurance of athletes (Sabbah, 2005).

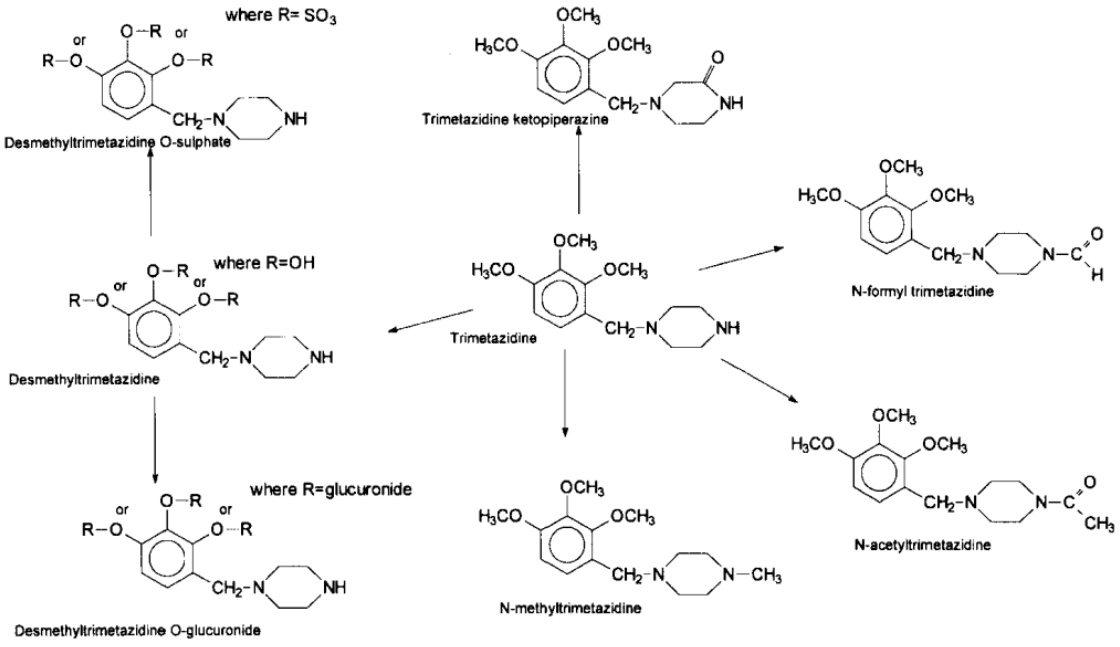

Figure 10: Metabolic Pathway of Trimetazidine
Source: Jackson, P.J.; Brownsill, R.D.; Taylor, A.R.; Resplandy, G.; Walther, B.; Schwietert, H.R. Identification of Trimetazidine Metabolites in Human Urine and Plasma *Xenobioteca* **1996** 26(2): pg 228

A previous GC-MS method developed for the analysis of trimetazidine was done by *Belal et al.* (2014); before this one other method by *Fay et. al.* was performed in 1989. These were the only two scientific articles that developed a GC-MS method

for the analysis of trimetazidine. Other methods involved using High Performance Liquid Chromatography-Photo Diode Array Detector (HPLC-DAD) (El-Alfy, 2019), Liquid Chromatography-Electron Spray Ionization Mass Spectrometry (LC/ESI-MS) (Jiao, 2007), and Gas Chromatography-Mass Spectrometry-Nitrogen Phosphate Detector(GC-MS-NPD) (Jerak, 2015; Sigmund, 2014).

In *Belal et. al.* the GC-MS instrument parameter were the same as TRI-M1 that is mentioned in the Results and Discussion section below. The authors were able to analyze a concentration range of 100-600 $\mu g*mL^{-1}$(Belal, 2014). The authors reported a LOD of 22.5 $\mu g*mL^{-1}$ and a LOQ of 74.9 $\mu g*mL^{-1}$ (Belal, 2014). One limit of their method, was that the authors did not test SIM, instead only using TIC, and they did not test an internal standard.

In *Fay et. al.* a similar method was used as Belal et. al. but they were testing trimetazidine after spiking blood and urine samples. The authors were able to get a linear range of 1-200 $ng*mL^{-1}$ in blood and 0.5-100 $\mu g*mL^{-1}$ in urine (Fay, 1989). At a concentration of 100 $ng*mL^{-1}$ in blood and in plasma with an internal standard, they had a precision of 3.4% and 4.5% respectively and at a concentration of 5 $\mu g*mL^{-1}$ in urine with an internal standard they had a precision of 0.3% (Fay, 1989). Limits of detection and quantitation were not recorded.

Experimental

External Calibration

Trimetazidine stock solution was prepared by dissolving the powder in methanol. The calibration solutions were prepared by dilution of aliquots of the

trimetazidine stock solution with methanol to reach the concentration range 0.025-0.1 $mg*mL^{-1}$. The solutions were chromatographed using the GC-MS. The MS was performed with TIC and SIM (m/z=166 Da and 181 Da). The peak areas of trimetazidine were plotted against the concentration to obtain the calibration curve. Excel was used to obtain linear regression parameters, such as the correlation coefficient and standard deviations of the slope, intercept, and residuals.

Internal Standard Calibration

Propranolol was selected as the internal standard. Propranolol stock solution was prepared by dissolving standard powder in methanol. Trimetazidine calibration solutions were spiked with 0.1 $mg*mL^{-1}$ of propranolol. The solutions were chromatographed using the GC-MS. The ratio of the instrument response of trimetazidine/propranolol was plotted against the ratio of the concentration of trimetazidine/propranolol to obtain the calibration curve.

Application to Urine and Extraction Method

Synthetic urine samples were spiked with 1 $mg*mL^{-1}$ trimetazidine; 3 mL of the urine samples were adjusted to a pH of approximately 14 with 22% sodium hydroxide, and the mixture was extracted with trichloromethane. The organic layer was evaporated, and the residue was reconstituted using 1 mL of HPLC grade methanol. The organic layer was analyzed by GC-MS.

Results and Discussion

Method Development

To start, a method from *Belal et. al* (2014) was used. The method that had a successful elution of trimetazidine was helium carrier gas flow rate of 0.9 mL/min and a column head pressure of 10 psi. The inlet temperature was 250°C and the auxiliary heater at 280°C. These parameters were kept the same as the temperature program was changed in order to improve the retention time.

Table 9: Temperature Programs and Retention Times for the Method Development of Trimetazidine using TIC at 0.1 mg*mL^{-1}

Method	Temperature Program			Retention Time of Trimetazidine
	Ramp (°C.min^{-1})	Temperature (°C)	Hold (min)	
TRI-M1		110	1	17.3 minutes
	7	180	3	
	15	280	1	
TRI-M2		110	1	14.0 minutes
	10	180	3	
	20	280	1	
TRI-M3		110	1	9.5 minutes
	15	180	3	
	20	280	1	

These retention times were overlaid in **Figure 11**. Using **Equation 1 and 2**, the number of theoretical plates was calculated for each of the methods and is presented in **Table 10**. Similar to the toremifene, as the retention time was decreased, the peaks got taller. However, the HETP increased instead of decreased. This happened because while the retention times were decreasing the width of the peak was remaining fairly constant as seen in **Figure 11.** While the optimum method would have been the first method, TRI-M1, it was not realized at the time of method development. The final method, TRI-M3, was used for the rest of the analysis of trimetazidine.

Table 10: Theoretical Plates and HETP for Trimetazidine

Method	Theoretical Plates	HETP (meters)
TRI-M1	781000	$3.8*10^{-5}$
TRI-M2	681000	$4.4*10^{-5}$
TRI-M3	436708	$6.9*10^{-5}$

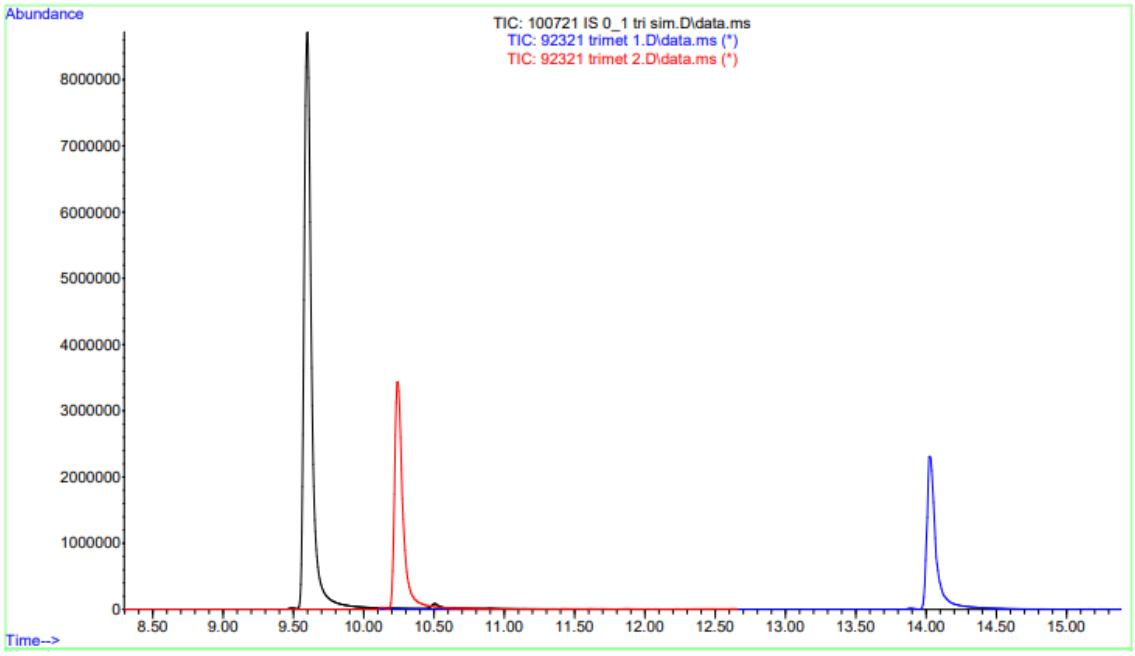

Figure 11: Trimetazidine Method Development Overlay using TIC at 0.1 mg*mL^{-1}
TRI-M1 (blue), TRI-M2 (red), TRI-M3 (black)

The above data was all collected with using TIC. For the SIM that will be mentioned in the next section, m/z of 166 and 181 Da were chosen, depicted in **Figure 12**.

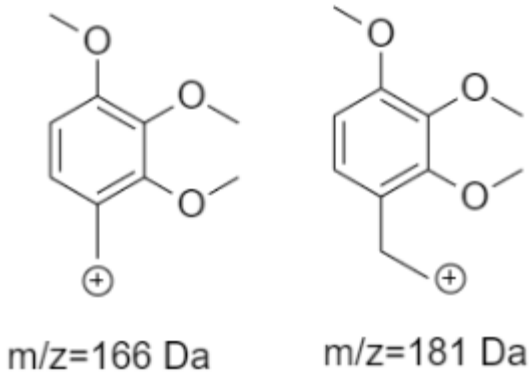

Figure 12: Trimetazidine Mass Spectrum Fragments m/z= 166 and 181 Da

Analytical Performance

The linearity for the procedure was analyzed by using different concentrations of trimetazidine. **Table 11** presents the statistical parameters of the linear regression including the concentration range, proportion of variance, LOD, and LOQ. The regression analysis showed good linearity for the SIM indicated by the r^2 value. The TIC method did not have as linear of a response as the SIM

Table 11: External Standard TIC and SIM Linear Regression Analytical Parameters for Trimetazidine

Parameter	Trimetazidine TIC	Trimetazidine SIM m/z= 166 and 181
Concentration range ($mg*mL^{-1}$)	0.0250-0.0750	0.0500-0.100
Correlation coefficient (r^2)	0.973	0.996
Limit of detection (LOD) ($mg*mL^{-1}$)	0.0297	0.0155
Limit of quantitation (LOQ) ($mg*mL^{-1}$)	0.0891	0.0464

Due to the steeper slope demonstrated in **Figure 13**, the TIC showed a higher level of sensitivity compared to SIM.

Using the SIM was generally more selective than TIC. Since it was only scanning for the selected m/z, there was less of a noise level. And as mentioned in the introduction, the LOD and LOQ are also related to the signal-to-noise ratio. Also, there was less baseline rising that can affect the peak area.

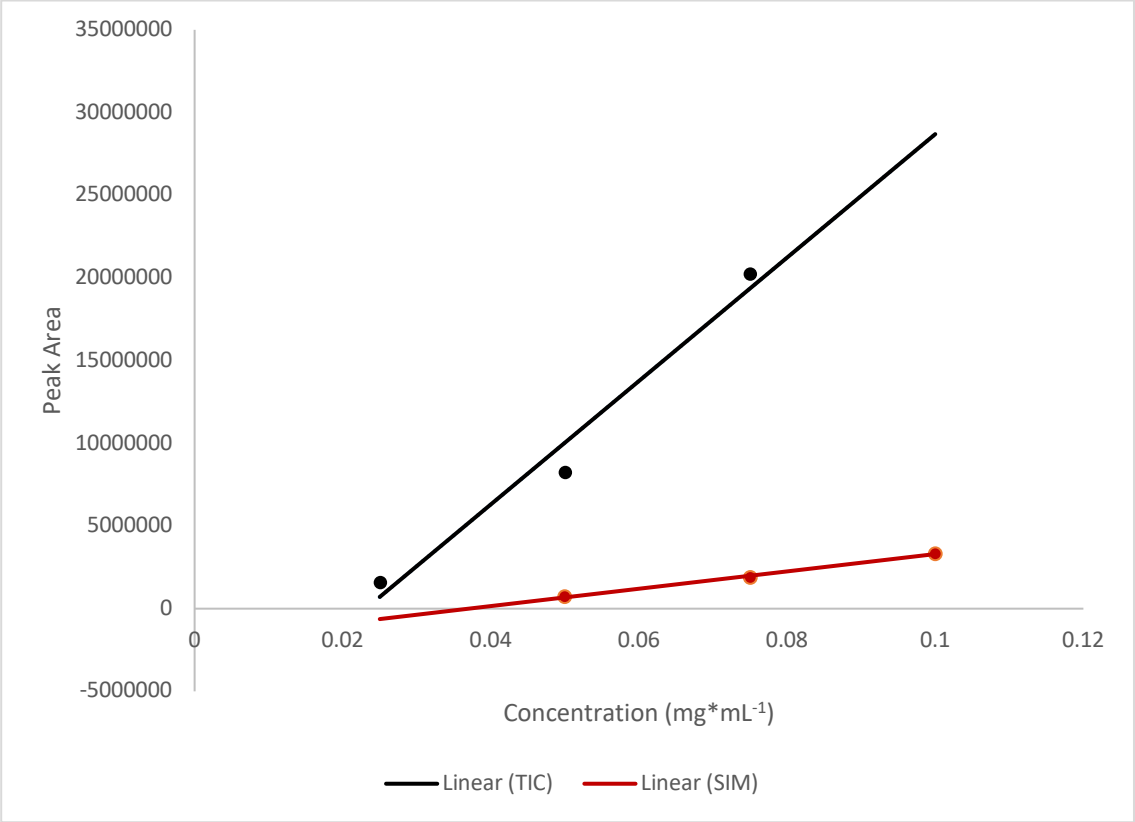

Figure 13: Trimetazidine TIC and SIM Linear Regression

Internal Standard

Using the same GC method as mentioned previously, propranolol eluted at approximately 11 minutes, as seen in **Figure 14**.

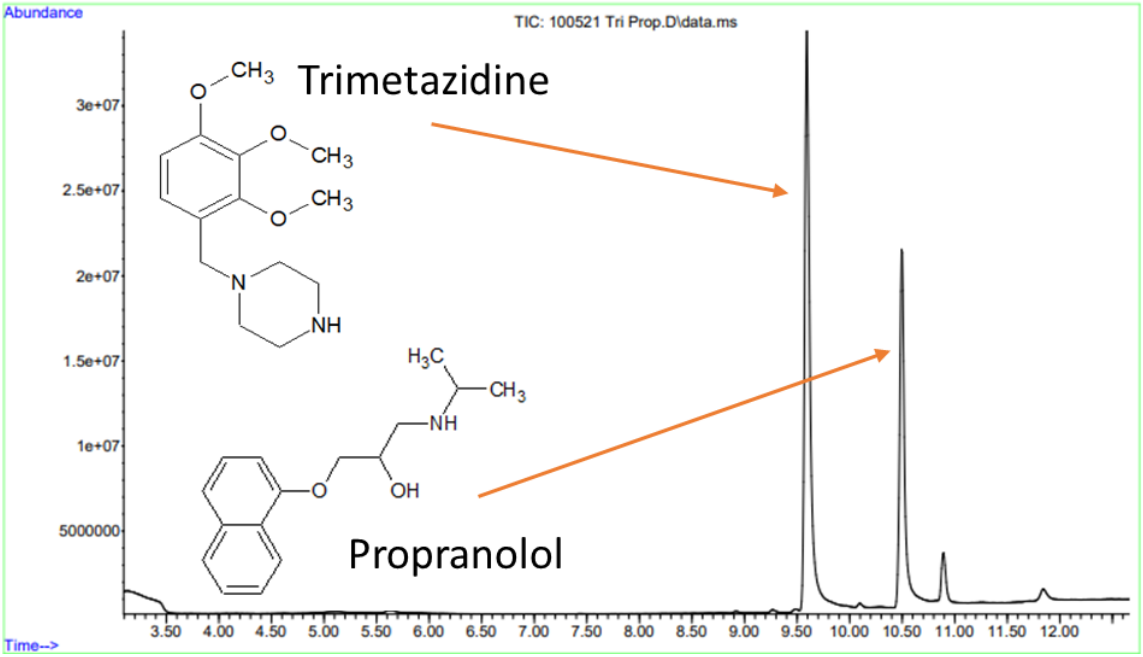

Figure 14: Gas Chromatogram of Trimetazidine and Propranolol

There was sufficient resolution between the trimetazidine (retention time 9.597 minutes) and the propranolol (retention time 10.502 minutes) peaks, with a calculated resolution of 16.7.

As before, the linearity was analyzed by using different concentrations of trimetazidine spiked with 0.1 $mg*mL^{-1}$ of propranolol. **Table 12** presents the same analytical parameters as **Table 11**. The LOD and LOQ in **Table 12** were calculated as a ratio of the concentration of trimetazidine and propranolol. Since the concentration of propranolol was 0.1 $mg*mL^{-1}$ for all samples, the LOD for trimetazidine was 0.01474 $mg*mL^{-1}$ and 0.05204 $mg*mL^{-1}$ for TIC and SIM respectively. The LOQ for trimetazidine was 0.04421 $mg*mL^{-1}$ and 0.1561 $mg*mL^{-1}$ for TIC and SIM respectively. These values were also recorded in **Table 12.**

Table 12: Trimetazidine with Internal Standard Linear Regression Analytical Parameters

Parameter	Trimetazidine/Propranolol TIC	Trimetazidine/Propranolol SIM m/z= 181 and 72
Concentration range ($mg*mL^{-1}$)	0.250-1.00	0.500-1.50
Correlation coefficient (r^2)	0.992	0.980
Limit of detection (LOD) ($mg*mL^{-1}$)	0.147	0.520
Limit of quantitation (LOQ) ($mg*mL^{-1}$)	0.442	1.56
	Trimetazidine TIC	Trimetazidine SIM
Limit of detection (LOD) ($mg*mL^{-1}$)	0.0147	0.0520
Limit of quantitation (LOQ) ($mg*mL^{-1}$)	0.0442	0.156

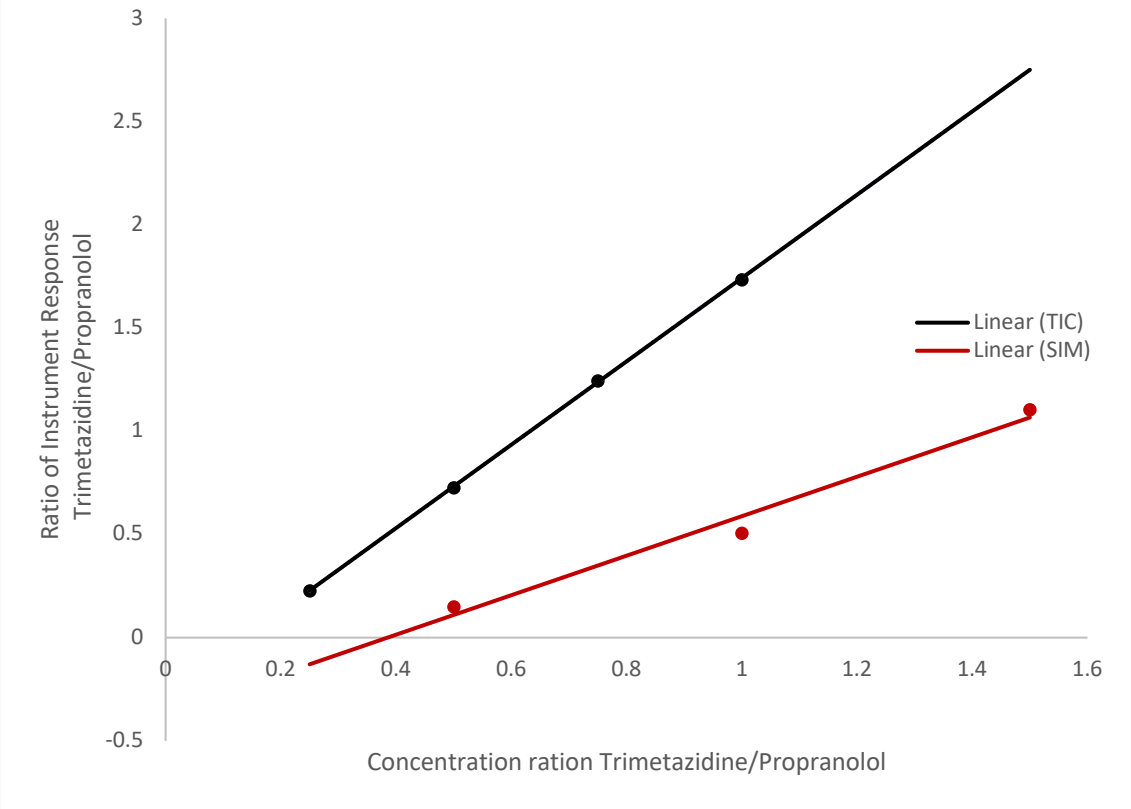

Figure 15: Trimetazidine/Propranolol TIC and SIM Linear Regression

In **Figure 15**, the TIC showed a steeper slope and therefore was a more sensitive method compared to the SIM with an internal standard.

Method Validation

Table 13: Trimetazidine TIC Precision and Accuracy

Labeled Concentration ($mg*mL^{-1}$)	Found Concentration ± Standard Deviation ($mg*mL^{-1}$)	%RSD	$\%E_r$
Intra-day (Repeatability)			
0.05	0.048 ± 0.001	2.87%	3.71%
0.075	0.076 ± 0.0008	1.07%	1.80%
0.1	0.103 ± 0.0007	0.71%	3.08%
Inter-day (Intermediate Precision)			
0.05	0.051 ± 0.003	7.21%	2.76%
0.075	0.072 ± 0.005	6.94%	3.68%
0.1	0.101 ± 0.002	2.55%	1.38%

n=5

Table 14: Trimetazidine SIM Precision and Accuracy

Labeled Concentration ($mg*mL^{-1}$)	Found Concentration ± Standard Deviation ($mg*mL^{-1}$)	%RSD	$\%E_r$
Intra-day (Repeatability)			
0.05	0.050 ± 0.0003	0.77%	0.74%
0.075	0.075 ± 0.001	2.01%	0.65%
0.1	0.101 ± 0.0008	0.86%	1.00%
Inter-day (Intermediate Precision)			
0.05	0.051 ± 0.0007	1.43%	1.77%
0.075	0.073 ± 0.002	2.77%	2.35%
0.1	0.101 ± 0.001	1.26%	0.88%

n=5

In order to test the repeatability, three different concentrations of toremifene were tested with both TIC (**Table 13**) and SIM (**Table 14**) a total of five times in sequence. The intermediate precision was also test by running the same concentration on three different days.

The $\%E_r$ values were found to be less than 5% which indicates that the proposed method was accurate. The %RSD values were found to be less than 2% which complies with the ICH guidelines and indicates that the proposed method was precise.

The TIC instrumental method had a higher LOD and LOQ compared to the SIM. As such, the 0.05 $mg*mL^{-1}$ and 0.075 $mg*mL^{-1}$ concentrations were below the LOQ for TIC. This reflects in the precision and accuracy of these two concentrations seen in **Table 13**.

The robustness (Appendix B) of the method was tested by intentionally varying the temperature program, inlet temperature, and auxiliary temperature by 2°C. These changes visually did not significantly change the instrument response of trimetazidine.

Urine Extraction

Before the trimetazidine could be extracted using chloroform, it had to be converted from the salt form to the free base form which is soluble in organic solvents. To do this, sodium hydroxide was added until the pH reached approximately 14. Trimetazidine is dibasic with pKa of 4.54 and 9.14 (Reymond, 1999). Generally, increasing the pH above the pKa of the compound leads to the predominance of the unionized free base form. Here the pH was increased to a little less than 5 pH units above the highest pKa of trimetazidine. This would mean approximately 99.99% of the trimetazidine was in its free base form according to the Henderson-Hasselbalch equation (Harris, 2010).

$$pH = pKa + log\frac{[base]}{[acid]}$$

Equation 9: Henderson-Hasselbalch Equation
Where the [base] is the concentration of unionized free base form and the [acid] is the concentration of the ionized salt form.

The amount of chloroform needed to extract trimetazidine from urine was optimized. After the pH was adjusted, the trimetazidine was extracted with 3 mL, 5 mL, 7 mL, and 10 mL of chloroform. The amount extracted was determined by comparing the peak area of the samples using TIC with the peak areas of the calibration standards. The results are recorded in **Table 15**.

Table 15: Extraction Optimization of Trimetazidine from Urine

Amount of Chloroform (mL)	3.05	5.00	6.98	10.09
Percent Trimetazidine Extracted	21.5%	28.7%	35.6%	37.1%

To determine the extraction efficiency the extraction was repeated 5 times on 1 mg*mL^{-1} trimetazidine spiked 3mL urine samples. Each sample was treated the same as mentioned in the Experimental section. The extraction efficiency was assessed by comparing peak area of the extracted solution to the peak area of the calibration standards. The average extraction efficiency was found to be 37% using 7 mL of chloroform, the percent extracted for each sample is recorded in **Table 16**.

Table 16: Extraction Efficiency of Trimetazidine from Urine using 7 mL of Chloroform

Trial	TIC	SIM	Average
1	27.6%	27.6%	27.6%
2	48.4%	33.0%	40.7%
3	35.6%	20.3%	28.0%
4	56.0%	49.2%	52.6%
5	45.0%	31.7%	38.3%
Average	42.5%	32.4%	37.5%
Standard Deviation	11.1	10.6	10.3
%RSD	26.1	32.8	27.6

V. Conclusion

Hormone and metabolic modulators account for approximately 8% of the recorded adverse analytical findings each year, yet there is limited analysis of toremifene and trimetazidine using GC-MS. The focus of this research was to develop simple, sensitive, economic, and fast analytical methods for the determination of toremifene and trimetazidine.

Toremifene is a SERM, that has not been detected by the WADA in the last 5 years. Toremifene had a final retention time of just under 16 minutes. The proposed methods using TIC and SIM reached a linearity correlation coefficient of 0.99 with a LOD of 0.015 $mg*mL^{-1}$ and LOQ of 0.045 $mg*mL^{-1}$. The method using SIM did have a higher slope (higher level of sensitivity) and a higher level of accuracy and precision. For toremifene, adding an internal standard did not significantly improve the analytical parameters.

Trimetazidine accounts for approximately 2% of the AAF for hormone and metabolic modulators each year. For the proposed method of trimetazidine using TIC it reached a linearity correlation coefficient of 0.9735 with a LOD of 0.030 $mg*mL^{-1}$ and LOQ of 0.089 $mg*mL^{-1}$. Using SIM did improve the parameters, so that a linearity of 0.996 with a LOD of 0.015 $mg*mL^{-1}$ and LOQ of 0.046 $mg*mL^{-1}$ was reached. While the TIC method did have a higher level of sensitivity, the SIM method had higher levels of accuracy and precision. Similar to the toremifene, adding an internal standard did not significantly improve the analytical parameters. Furthermore, a proposed method was applied for the determination of trimetazidine in urine using liquid extraction with a 37% extraction efficiency.

Future Research

In this thesis project, toremifene was not analyzed within a biological fluid. Further research will be need in order to extract and analyze toremifene in a biological matrix such as plasma or urine. Also, this research done without the presence of metabolites. More research could be done to ensure that GC-MS can distinguish toremifene from the metabolites that bind to plasma proteins.

The method presented in this thesis had a low urine extraction. Further research could be used to improve the extraction, such as using a different organic solvent or using an internal standard. While trimetazidine does not undergo biotransformation to a large extent, this research did not analyze trimetazidine in the presence of its metabolites. Further experimentation may be needed to analyze trimetazidine in the presence of metabolites.

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APPENDICES

[Appendix A: Linear Regression Data]

Table 17: External Standard of Toremifene Linear Regression Analytical Parameters

Parameter	Toremifene TIC	Toremifene SIM m/z=58
Concentration range ($mg*mL^{-1}$)	0.025-0.075	0.025-0.1
Correlation coefficient (r^2)	0.9932	0.9909
Slope (b)	51519520	198604128
Intercept (a)	-5723914	-3938936
Standard error of slope (S_b)	427771332	13428569
Standard error of intercept (S_a)	2309916	919391
Standard error of residuals ($S_{y/x}$)	1512194	750680
Limit of detection (LOD) ($mg*mL^{-1}$)	0.015	0.015
Limit of quantitation (LOQ) ($mg*mL^{-1}$)	0.045	0.046

Table 18: Internal Standard of Toremifene Linear Regression Analytical Parameters

Parameter	Toremifene/Tamoxifen TIC	Toremifene/Tamoxifen SIM m/z=58
Concentration range ($mg*mL^{-1}$)	0.025-0.15	0.025-0.15
Correlation coefficient (r^2)	0.9870	0.9758
Slope (b)	0.6932	0.5694
Intercept (a)	-0.09479	0.1163
Standard error of slope (S_b)	0.05636	0.06330
Standard error of intercept (S_a)	0.05319	0.05974
Standard error of residuals ($S_{y/x}$)	0.05411	0.06078
Limit of detection (LOD) ($mg*mL^{-1}$)	0.2558	0.3497
Limit of quantitation (LOQ) ($mg*mL^{-1}$)	0.7673	1.049

Table 19: External Standard of Trimetazidine Linear Regression Analytical Parameters

Parameter	Trimetazidine TIC	Trimetazidine SIM m/z= 166 and 181
Concentration range ($mg*mL^{-1}$)	0.025-0.075	0.05-0.1
Correlation coefficient (r^2)	0.9735	0.9964
Slope (b)	373100000	52400000
Intercept (a)	-8618000	-1940000
Standard error of slope (S_b)	61590000	3129000
Standard error of intercept (S_a)	3326000	243200
Standard error of residuals ($S_{y/x}$)	2177000	110600
Limit of detection (LOD) ($mg*mL^{-1}$)	0.030	0.015

Limit of quantitation (LOQ) (mg*mL^{-1})	0.089	0.046

Table 20: Internal Standard of Trimetazidine Linear Regression Analytical Parameters

Parameter	Trimetazidine/Propranolol TIC	Trimetazidine/Propranolol SIM m/z= 181 and 72
Concentration range (mg*mL^{-1})	0.25-1	0.5-1.5
Correlation coefficient (r^2)	0.9917	0.9795
Slope (b)	2.065	0.9564
Intercept (a)	-0.2625	-0.3683
Standard error of slope (S_b)	0.1334	0.1382
Standard error of intercept (S_a)	0.09130	0.1493
Standard error of residuals ($S_{y/x}$)	0.07455	0.09775
Limit of detection (LOD) (mg*mL^{-1})	0.1474	0.5204
Limit of quantitation (LOQ) (mg*mL^{-1})	0.4421	1.561

[Appendix B: Robustness]

Method TOR-M4 (Yellow in **Figure 22**) was changed to the following:

TOR-M4a (Black in **Figure 22**) Inlet temperature was changed to 247°C

TOR-M4b (Blue in **Figure 22**) temperature program was changed to initial temperature of 110°C for 1 minute, ramp up to 227°C at a rate of 20°C.min-1, hold at 227°C for 5 minutes, ramp up to 340°C at a rate of 30°C.min-1, hold at 340°C for 2 minutes.

TOR-M4c (Red in **Figure 22**) temperature program was change to initial temperature of 110°C for 1 minute, ramp up to 230°C at a rate of 20°C.min-1, hold at 230°C for 5 minutes, ramp up to 342°C at a rate of 30°C.min-1, hold at 342°C for 2 minutes.

TOR-M4d (Green in **Figure 22**) Auxiliary heater temperature was change to 278°C.

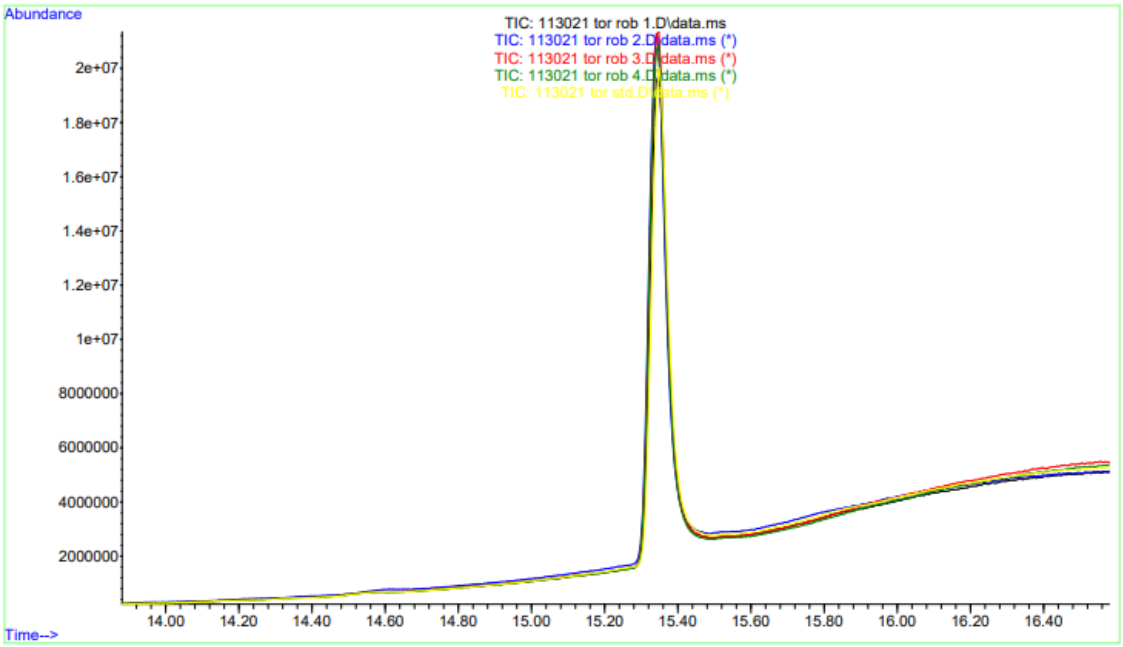

Figure 16: Testing the robustness of the developed method of analysis of Toremifene

Method TRI-M3 (Yellow in **Figure 23**) was changed to the following:

TRI-M3a (Black in **Figure 23**) inlet temperature was changed to 247°C.

TRI-M3b (Blue in **Figure 23**) temperature program was changed to initial temperature of 110°C for 1 minute, ramp up to 182°C at a rate of 15°C.min-1, hold at 182°C for 1 minute, ramp up to 280°C at a rate of 20°C.min-1, hold at 280°C for 1 minute.

TRI-M3c (Red in **Figure 23**) temperature program was changed to initial temperature of 110°C for 1 minute, ramp up to 180°C at a rate of 15°C.min-1, hold at 180°C for 1 minute, ramp up to 282°C at a rate of 20°C.min-1, hold at 282°C for 1 minute.

TRI-M3d (Green in **Figure 23**) Auxiliary heater temperature was change to 278°C.

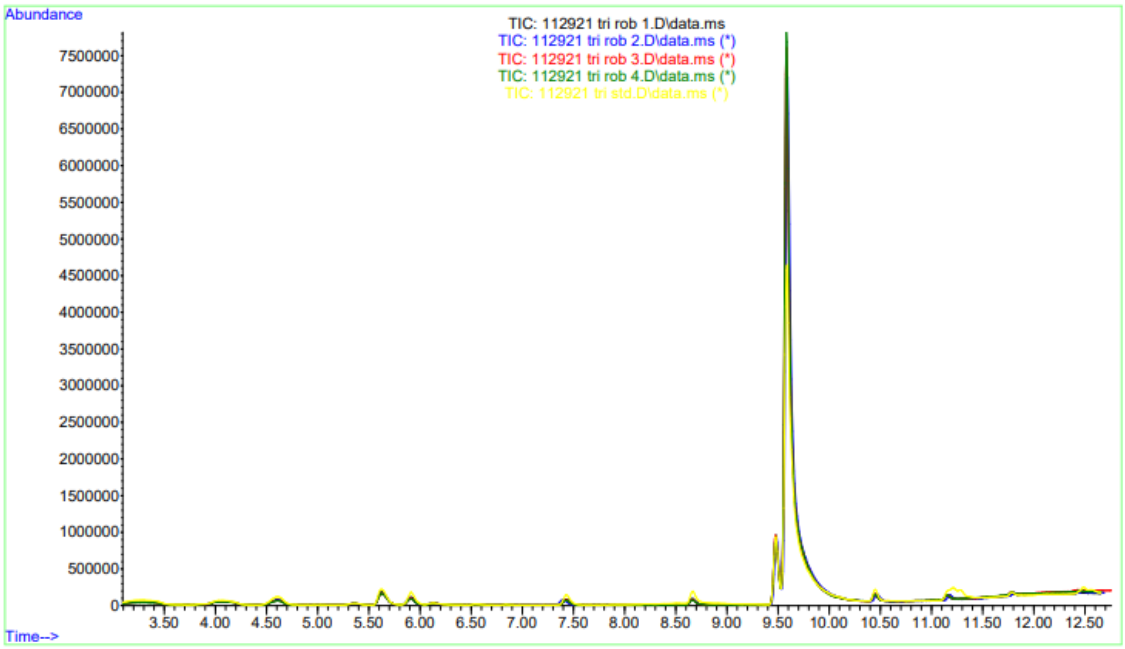

Figure 17: Testing the robustness of the developed method of analysis of Trimetazidine

[Appendix C: Mass Spectra]

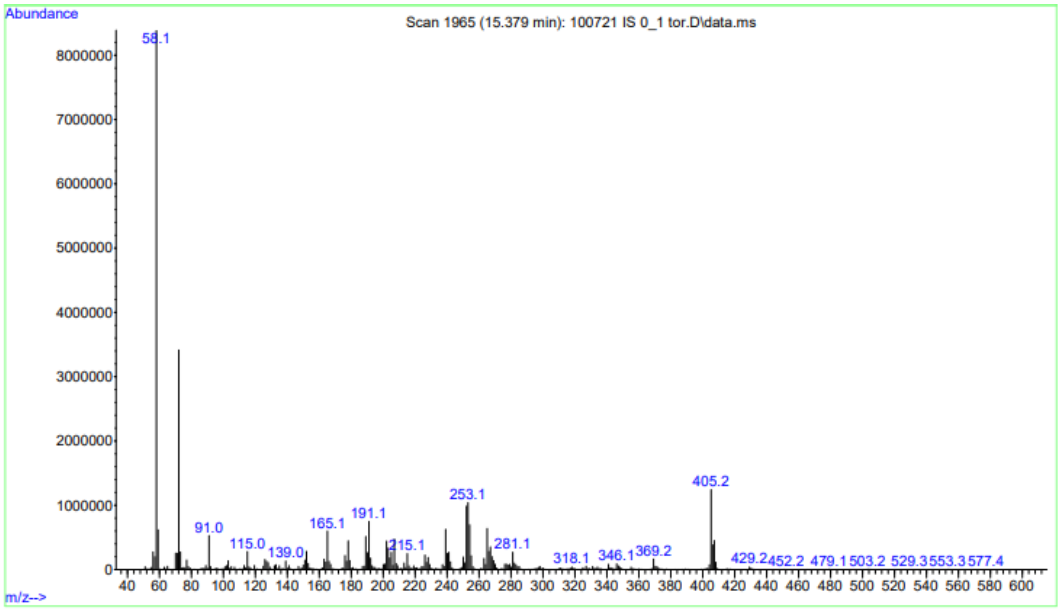

Figure 18: Electron Impact Mass Spectrum of Toremifene

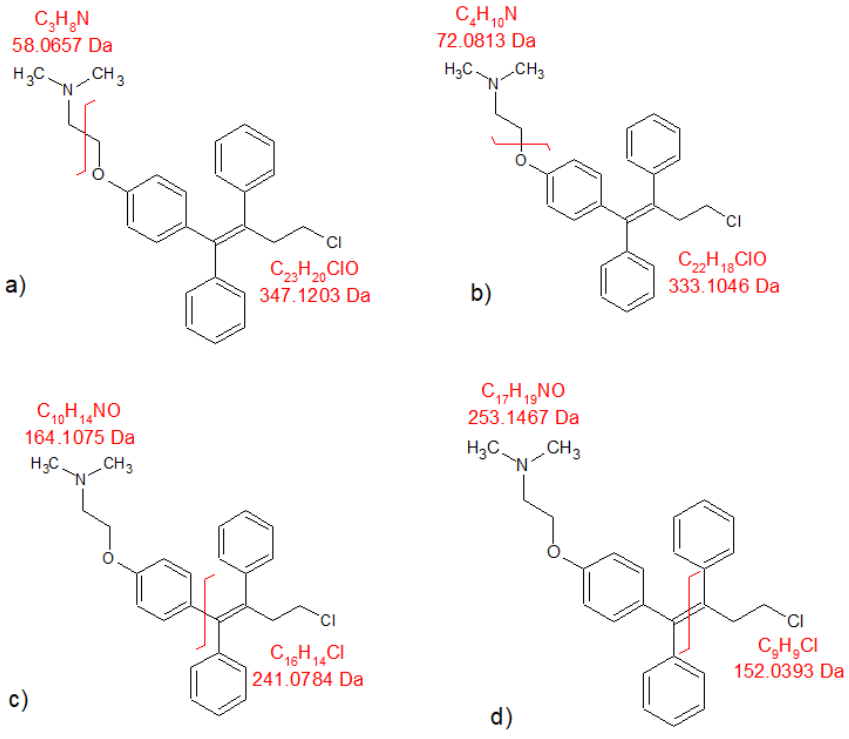

Figure 19: EI Mass Spectral Fragment Identifications for Toremifene

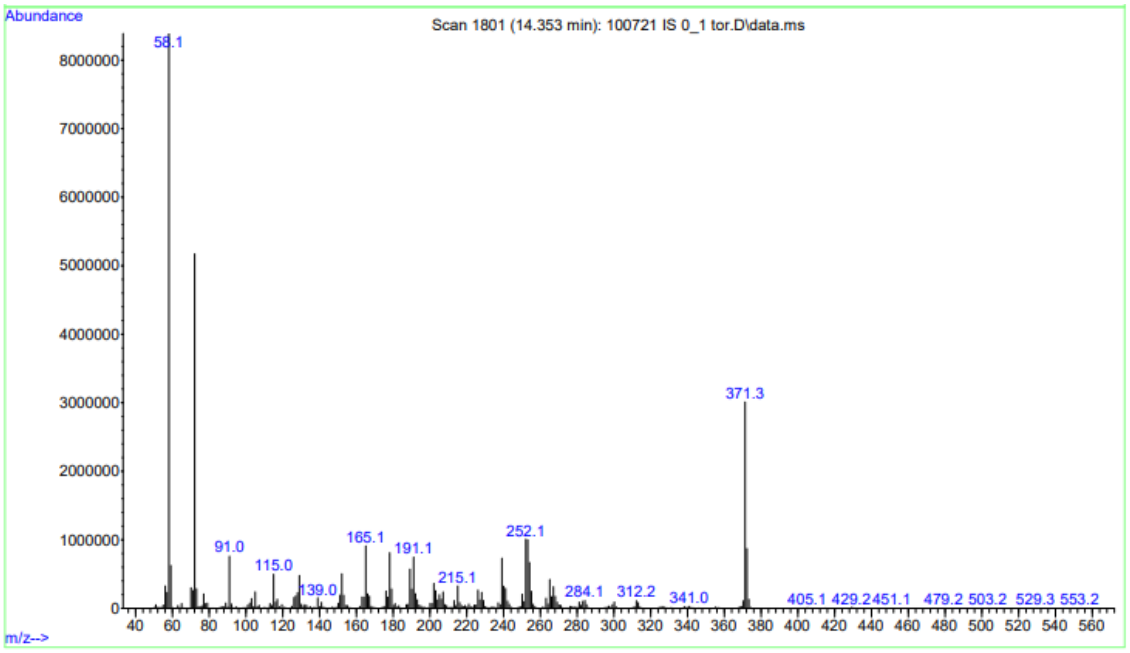

Figure 20: Electron Impact Mass Spectrum of Tamoxifen

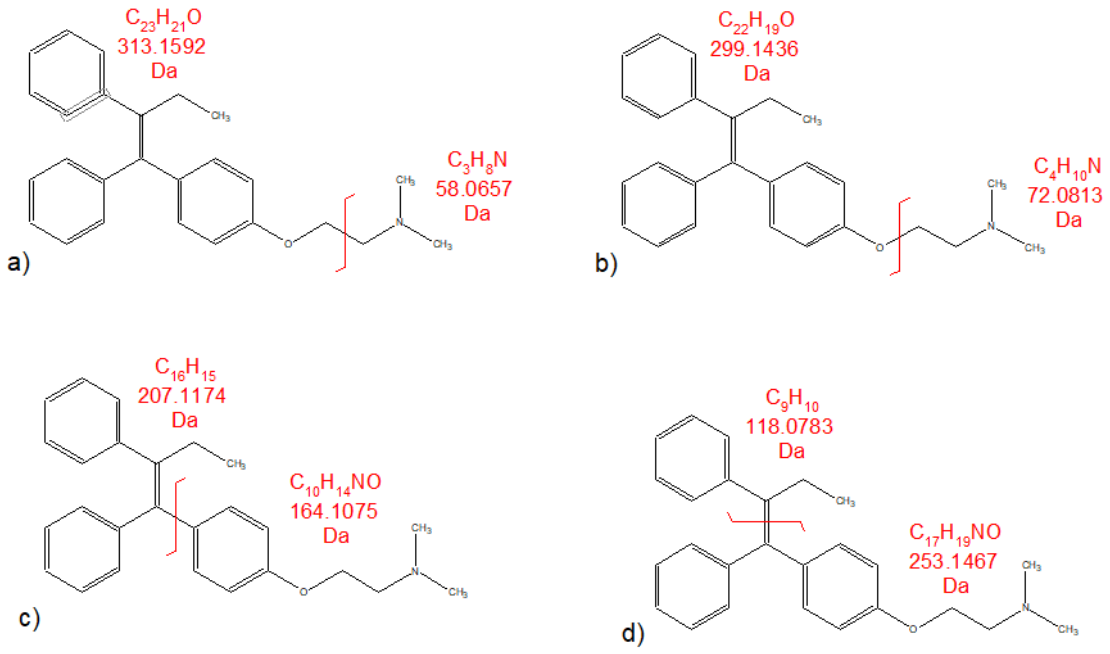

Figure 21: EI Mass Spectral Fragment Identifications for Tamoxifen

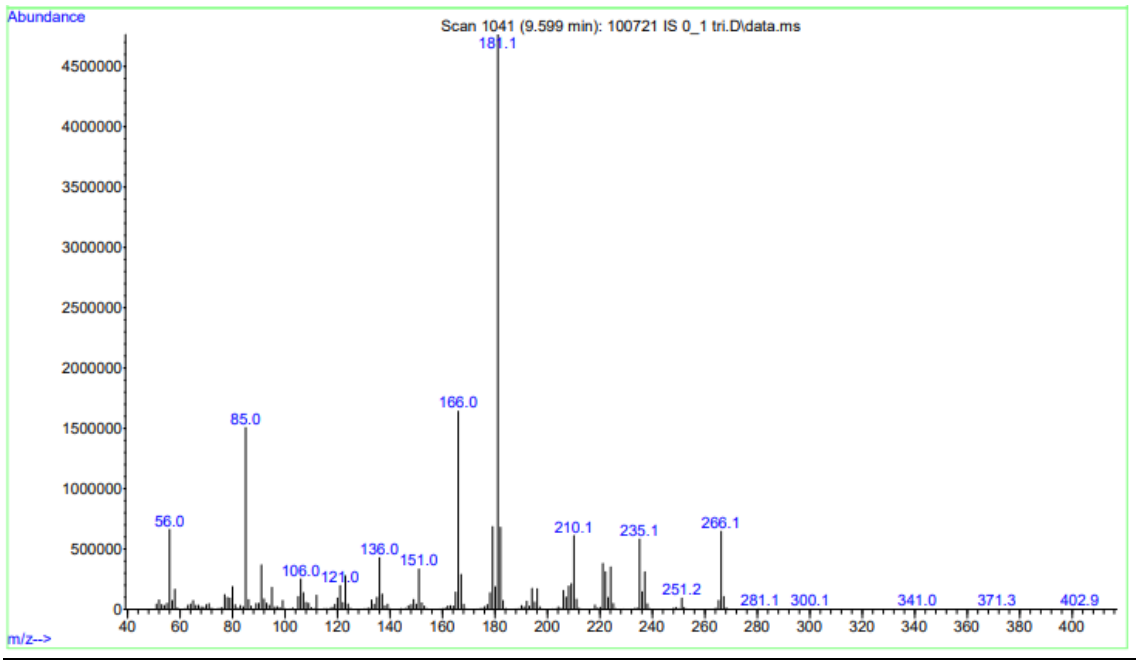

Figure 22: Electron Impact Mass Spectrum of Trimetazidine

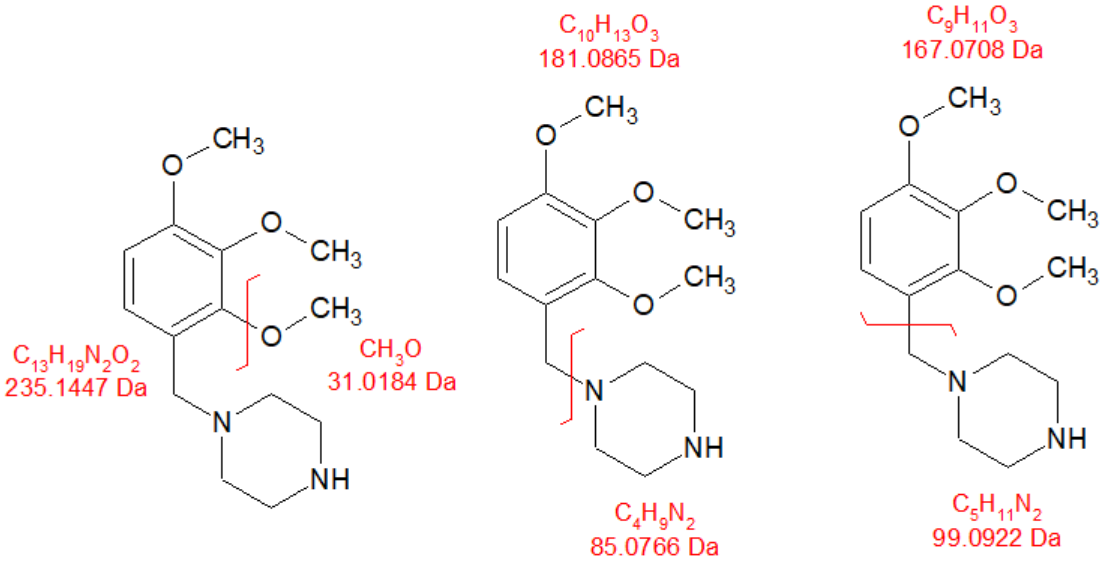

Figure 23: EI Mass Spectral Fragment Identifications for Trimetazidine

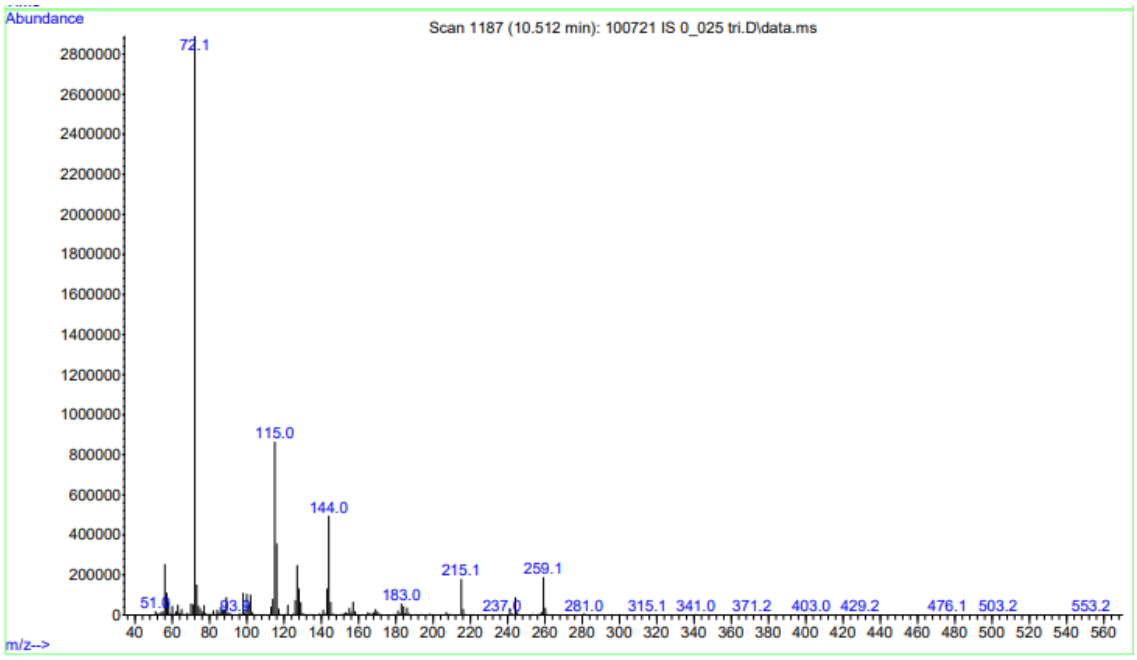

Figure 24: Electron Impact Mass Spectrum of Propranolol

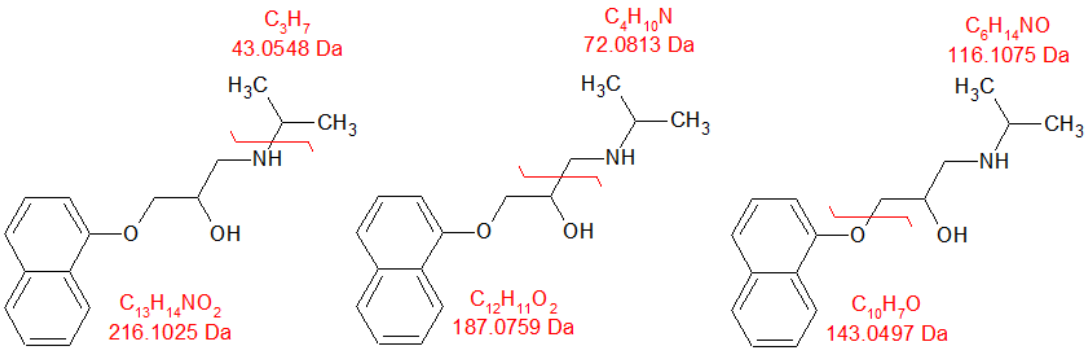

Figure 25: EI Mass Spectral Fragment Identifications for Propranolol