3.1 Abstract

The aim of the present study was to conduct an in vivo experiment on rats to study the degradation pattern of Cardiac Troponin I and its dependency on the cause of death for determining the postmortem interval. The rats were divided into three groups depending upon the cause of death (Control, Cardiotoxicity of Acebutolol, Asphyxia). The analysis involves extraction of the protein, separation followed by Western blotting for visualization. A characteristic degradation pattern of cTnI into lower molecular weight fragments with respect to time conclusively established that Cardiac Troponin I can be used as a suitable marker for the estimation of postmortem interval. Significant differences were observed in the degradation pattern of Cardiac Troponin I in different cases of death. This established that the method can also be used for determining if cardiac failure was the cause of death or not.
3.2 Introduction

In developing countries population explosion, poverty and increasing stress and strain in daily life, often lead to cases of suicides, homicides and accidents. With urbanization, rural areas are also not left aloof and this can be seen from the increasing incidence of suicides as well as of homicides.\(^1\) Hanging is a major mode for suicidal death and the prime reasons for it are:

(a) Easy availability of material  
(b) Minimum pain  
(c) Least time required  
(d) Almost 100% success in first attempt

Thus, a large number of suicides encountered are due to asphyxia death. The unhealthy life style and growing stress are the major reasons for the growing number of patients suffering from cardiovascular diseases. And so the demand of beta blockers has grown tremendously. Although beta blockers are used for treating hypertension but few of them when taken in overdose can be fatal. Acebutolol is one such beta-adrenoreceptor blocking agent that is considered one of the most toxic beta blockers when taken in overdose.\(^2\)

Considering the above mentioned factors, Acebutolol and Asphyxia were selected for this study.

Determination of postmortem interval is a common practice in medico-legal investigation. The commonly used methods are algor mortis, rigor mortis, livor mortis, putrefaction but they are affected by external conditions and thus the chance of producing erroneous results is high. Techniques based on biochemical changes offers advantage over physical methods since they are neither altered nor contaminated easily and rapidly.\(^3\,^6\) Despite the development of biochemical methods very few are
in practice today because of the time-consuming methodology and lack of standardized procedures.

The cardiac troponin (cTn) has been known as a marker of heart damage and myocardial cell death for more than 10 years. In toxicological studies, troponin has been established as a biomarker for drug-induced cardiac injury. Previous studies have suggested the possible application of cTnI in the postmortem diagnosis of acute myocardial infarction, cardiac contusions, and for the estimation of postmortem interval. But due to the lack of data and standardized procedures, a general agreement has not been established to use cardiac troponin in forensic casework.

TnI is present both in skeletal as well as cardiac muscles. But, since heart is a well protected organ, the effect of external conditions is less as compared to the skeletal muscles. Therefore, cTnI was chosen as the material of study. It undergoes degradation with time which proceeds in an orderly manner leading to the appearance of a wide diversity of short fragments of it. This disappearance of intact cardiac troponin into lower molecular weight fragments can be easily monitored by western blotting.

The present study investigated the degradation pattern of cTnI with respect to time. A comprehensive analysis of cardiac troponin I with regard to the cause of death (Cardiotoxicity due to Acebutolol, Asphyxia and Control) was studied.
3.2.1. Asphyxia

The word asphyxia is derived from the Greek and means 'pulseless'. It is a condition of severely deficient supply of oxygen to the body that arises from being unable to breathe normally. When a person is subjected to asphyxia, unconsciousness generally occurs in 2 - 3 mins, and death in 4 - 5 mins.\textsuperscript{14}

Death may however be hastened by factors such as: struggling - the increased physical activity uses up available oxygen much faster, pre-existing respiratory disease or poor general state of health from any cause.
3.2.1.1 Classification of Asphyxia

Asphyxia can be classified into two groups mechanical and non-mechanical. The examples of each group are listed in Fig.3.1.
3.2.1.2 Signs of Asphyxia

Classical signs of Asphyxia have been described for many years that consist of petechial hemorrhages of the face, engorgement with blood (congestion), fluid overload of the tissue (edema), blue discoloration of the skin (cyanosis), and engorgement of the right side of the heart with blood. However, it is now recognized that these are in no ways sensitive or specific for asphyxia. Thus, as there are no diagnostic pathological features of acute asphyxia, autopsies may have negative or non-specific findings that might only suggest the possibility.

The petechial hemorrhages may also occur on the pleural surface of the lungs and on the surface of the heart. However they are non-specific and also occur in congestive heart diseases, drug overdoses when mechanical obstruction is not necessarily a feature. Pulmonary edema occurs when the death is prolonged but the degree of edema should not be taken as an indication of time elapsed since death.

3.2.1.3 Forensic Aspects

An increasing death rate as a result of violence constitutes a large group in medicolegal autopsies. Specially, deaths due to asphyxia are one of the most important causes in violence deaths. Among fatalities that are subjected to medico-legal autopsies, asphyxia-related deaths account for a significant number of cases.

In a study done by Department of Forensic Medicine, Trakya University, Edirne, Turkey for the time period of January 1984 to October 2004, there were 134 asphyxial deaths autopsied by them. In another study in India, 37 cases of violent mechanical homicidal asphyxia deaths were reported.
during the years 2000 to 2004 in Jamnagar, Gujarat. It constituted about 20.67% of the total 179 homicidal deaths brought during the period.\(^{16}\)

### 3.2.2 Acebutolol

The use of beta-blocking agents is rapidly increasing because of their significant beneficial effects in the management of hypertension, angina pectoris, and cardiac arrhythmias.

Acebutolol HCl is a selective, hydrophilic beta-adrenoreceptor blocking agent with mild intrinsic sympathomimetic activity for use in treating patients with hypertension and ventricular arrhythmias. It is marketed in capsule form for oral administration.\(^{17,18}\)

Acebutolol HCl is a white or slightly off-white powder freely soluble in water, and less soluble in alcohol. Chemically it is defined as the hydrochloride salt of:

\[
\text{N-[3-acetyl-4-[2-hydroxy-3-(propan-2-ylamino)propoxy]phenyl] butanamide}
\]

![Fig.3.2. Chemical Structure of Acebutolol Hydrochloride](image)
3.2.2.1 Pharmacodynamics

β₁-cardioselectivity has been demonstrated in animal studies under laboratory conditions. In anesthetized dogs and cats, acebutolol is more potent in antagonizing isoproterenol-induced tachycardia (β₁) than in antagonizing isoproterenol-induced vasodilatation (β₂). In guinea pigs and cats, it is more potent in antagonizing this tachycardia than in antagonizing isoproterenol-induced bronchodilatation (β₂). ISA of acebutolol has been demonstrated in catecholamine-depleted rats by tachycardia induced by intravenous administration of this agent. A membrane-stabilizing effect has been detected in animals, but only with high concentrations of acebutolol.\textsuperscript{17, 18}

Clinical studies have demonstrated β₁-blocking activity at the recommended doses by:

a) reduction in the resting heart rate and decrease in exercise-induced tachycardia;

b) reduction in cardiac output at rest and after exercise;

c) reduction of systolic and diastolic blood pressures at rest and postexercise;

d) inhibition of isoproterenol-induced tachycardia.

The β₁-selectivity of acebutolol has also been demonstrated on the basis of the following vascular and bronchial effects:

Vascular Effects: Acebutolol has less antagonistic effects on peripheral vascular β₂ receptors at rest and after epinephrine stimulation than nonselective β-antagonists.
Bronchial Effects: In single-dose studies in asthmatics examining effects of various beta-blockers on pulmonary function, low doses of acebutolol produce less evidence of broncho constriction and less reduction of beta2 agonist, bronchodilating effects, than nonselective agents like propranolol but more than atenolol.

3.2.2.2 Pharmacokinetics and Metabolism

Acebutolol is well absorbed from the gastro intestinal tract. It is subject to extensive first-pass hepatic biotransformation, with an absolute bioavailability of approximately 40% for the parent compound. The major metabolite, an N-acetyl derivative (diacetolol), is pharmacologically active. This metabolite is equipotent to acebutolol and in cats is more cardioselective than acebutolol; therefore, this first-pass phenomenon does not attenuate the therapeutic effect of acebutolol.

The plasma elimination half-life of acebutolol is approximately 3 to 4 hours, while that of its metabolite, diacetolol, is 8 to 13 hours. The time to reach peak concentration for acebutolol is 2.5 hours and for diacetolol, after oral administration of acebutolol, 3.5 hours.

Elimination via renal excretion is approximately 30% to 40% and by non-renal mechanisms 50% to 60%, which includes excretion into the bile and direct passage through the intestinal wall.\textsuperscript{17,18}
3.2.2.3 Toxicity of Acebutolol

The increasing use of beta-adrenoceptor antagonists appears to have resulted in more frequent reports of severe high dose intoxication. The adult therapeutic dose ranges from 200 mg to 1200 mg/day. There is a great variability in lethal and minimally toxic doses in acebutolol poisoning. The clinical features are well established. Cardiovascular suppression results in bradycardia, heart block and congestive heart failure. Bronchospasm and occasionally hypoglycemia can also occur. Coma and epileptic form seizures are often seen. Death has been caused by 400 mg of acebutolol. It is considered as one of the most toxic beta blockers and has resulted in fatalities. 17-19

3.3 Materials and Methods

3.3.1 Chemicals

Sodium chloride (FINAR Chemicals Ltd.), Sodium azide (SRL), Boehringer-Mannheim Complete Protease Inhibitor cocktail, Sodium dodecyl sulphate (Merck Ltd.), Sodium phosphate (FINAR Chemicals Ltd.), Tris (SRL), Ammonium persulfate (APS) (SRL), Nitrocellulose membrane (GenScript USA Inc), Glycerol (SD Fine), β- Mercaptoethanol (SRL), EDTA (FINAR Chemicals Ltd.), Bromophenol blue (Sigma-Aldrich), Glycine (FINAR Chemicals Ltd.), Acrylamide (SRL), TEMED (SRL), Sodium pentobarbital (Sigma- Aldrich), Acebutolol (SRL), A primary antibody specific for human cTnT (BiosPacific, Inc.), Tween 20 (FINAR Chemicals Ltd.), Horseradish peroxidase-labeled secondary antibody (antimouse IgG, BiosPacific, Inc.)
3.3.2 Instruments

3.3.2.1 Homogenizer: RQ 127 (Remi Motors, India)
3.3.2.2 Ultra High Speed Centrifugation machine: Remi Motors, India
3.3.2.3 Blotting Kit: C.B.S. Scientific Company
3.3.2.4 300 V Power Pac: C.B.S. Scientific Company

3.3.3 Animals and Experimental Groups

Female rats (n= 30) weighing 250-300 g were obtained from K. B. Institute of Pharmaceutical Education and Research, Gandhinagar, Gujarat, India.

They were housed in suspended wire cages approximately 10 cm above sawdust bedding trays with a 12-h light/12-h dark cycle in the animal housing facility. Temperature and relative humidity were controlled at 22°C and 55%, respectively. All animals were acclimatized to these conditions for one week with free access to standard laboratory rodent chow and drinking water ad libitum. All animals were cared for according to the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) and the protocol was approved by the K. B. Institute of Pharmaceutical Education and Research, Gandhinagar Ethical committee (Ref. No. KBIPER/2011/235).

The rats were divided into three groups depending upon the cause of death Group 1 (Control, n = 10), Group 2 (Asphyxia, n=10) and Group 3 (Acebutolol, n=10).
3.3.4 Animal Death and Tissue Sampling

The rats in Group 1 were anesthetized with Sodium pentobarbital (40 IP). The chest cavity was then quickly opened, and the heart was excised out. The rats in Group 2 (Asphxial Death) were sacrificed by clamping a tracheostomy tube and the rats in Group 3 (Acebutolol Death) were sacrificed by giving an oral lethal dose of Acebutolol (6620 mg/kg).

The sacrificed rats were incubated at 28°C for different time interval (0 hrs, 6 hrs, 12 hrs, 24hrs, 48 hrs, 72 hrs, 96 hrs and 100 hrs). The heart was then isolated and freezed at -80°C.

3.3.5 Sample Preparation

In forensic cases the amount and type of sample is very crucial since many a times the sample amount is very less. Thus, it is crucial to use an effective extraction method in order to obtain enough high quality uncontaminated sample of cTnI. The extraction of protein from tissue was optimized so as to stop or minimize the degradation of protein during the extraction process. The conventional method was used for extraction with slight modifications in the extraction buffer and an added step of carrying every step of the extraction process at low temperatures (1-4°C).

Frozen myocardial samples were cut into small pieces in sample trays placed on dry ice. 1 g of cardiac tissue was homogenized with 5 ml of ice cold extraction buffer (10 mM sodium phosphate, 100 mM Tris, 200 mM Sodium chloride, 0.1% sodium azide, 1 tablet in 50 ml of Complete Protease Inhibitor cocktail, ph 8.0). Samples are then centrifuged at 5000 g for 10 min. The supernatant was collected for analysis. Protein
concentrations were determined using Bradford Assay. The procedure yielded > 99% recovery of protein.

### 3.3.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS – PAGE)

SDS-PAGE is a method used for separating proteins according to their electrophoretic mobility. Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures secondary and non-disulfide–linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. The proteins migrate in response to an electrical field through pores in the gel matrix. The combination of gel pore size and protein charge, size and shape determines the migration rate of a protein.

![Schematic Illustration of Protein Fractionation by SDS – PAGE](image_url)
The SDS-PAGE was carried out according to the Laemelli protocol from 1970. The separating gel was prepared and allowed to polymerize on the bottom of the cast before pouring the stacking gel on top. Due to the higher acrylamide percentage in the separating gel, the proteins will be concentrated on the interface between the two gels resulting in increased protein resolution. After complete polymerization the chamber was assembled and running buffer is added into the well. The protein sample is mixed with the sample buffer and loaded onto the gel (5μl per/lane). The electrophoresis was carried out at 100 V for 1-2 hours, using Power Pac 300.

Table 3.1. Reagents Required for Preparing Various Buffers in SDS-PAGE

<table>
<thead>
<tr>
<th>Sample buffer</th>
<th>Running buffer</th>
<th>Polyacrylamide gel Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.125 M Tris-HCl (pH 6.8), 4 % (w/v) SDS, 20 % (v/v) Glycerol, 10 % (v/v) β-Mercaptoethanol, 0.01 % (w/v) Bromophenol blue</td>
<td>250 mM Tris, 1.92 M Glycine, 1 % SDS</td>
<td>30% Acrylamide, 10% SDS, 10% APS, TEMED, 1.5 M Tris (pH 8.8), H₂O</td>
</tr>
</tbody>
</table>
3.3.7 Western Blot

Western blot is a technique that is used to detect the expression of a specific protein in tissue homogenates or cell extracts after size separation by using gel electrophoresis. Proteins separated using SDS-PAGE before are transferred to a membrane where they are probed by antibodies specific to the target protein, Fig.3.4. Following a blocking step to prevent any nonspecific binding of antibodies to the surface of the membrane, the membrane is probed with a primary antibody that attaches to the antigen of interest. After a washing step, the membrane is incubated with a secondary antibody that is reactive toward the primary antibody. After probing with secondary antibody, the membrane is washed again and incubated with an appropriate enzyme substrate. One of the substrates used for protein detection is luminol-based and produce a chemiluminescent signal. Chemiluminescence is a chemical reaction that produces energy released in the form of light in the presence of horseradish peroxidase and a peroxide buffer. The intensity of the signal after development should correlate with the abundance of the antigen on the membrane.\textsuperscript{21}

Tissue homogenates after being size fractionated on 12% SDS-polyacrylamide gels were transferred to nitrocellulose membranes. Nonspecific binding sites were blocked by incubating the membranes in a blocking buffer [5.0% nonfat dry milk in Tris-buffered saline (TBS): 20 mM Tris HCl (pH 7.6), 137 mM NaCl] for 1 hr. A primary antibody specific for human cTnT (BiosPacific, Inc.) was diluted (1:1,000) in antibody buffer (1.0% nonfat dry milk in TBS) and incubated with the membranes for 2 hr on a rotating cylinder. The membranes were washed three times with Tween-TBS for 30 min. Horseradish peroxidase-labeled secondary antibody (antimouse IgG, BiosPacific, Inc.) was then incubated
with the membranes for 1 hr at a dilution of 1:3,000. The blot is developed using a colorimetric precipitating substrate.

![Schematic Illustration of the Western Blot Set up](image)

**Fig.3.4. Schematic Illustration of the Western Blot Set up**

### 3.3.8 Statistical Analysis

One way ANOVA was done on the three groups. ANOVA is used to compare the means of more than two samples. MS Excel was used to perform the ANOVA test.
3.4 Results and Discussion

The aim of this study was to use cTnI for determining postmortem interval. The results indicated a time dependent process of cTnI degradation. The degradation profile clearly showed a consistent degradation of the intact band of cTnI followed by the appearance of lower molecular weight degradation products with respect to time in all the three experimental groups (Fig. 3.5., Fig. 3.6., Fig. 3.7.).

A qualitative approach was followed in order to see whether the degradation of cTnI is dependent upon the cause of death. The rats were grouped in to three categories depending upon the cause of death. Fig.3.8. shows that rate of degradation of cTnI into lower molecular weight fragments with respect to time in the three different cases. It can be observed that in case of death due to Acebutolol poisoning, the intact cTnI fragmented at a much faster rate than in Control and Asphyxia group. Thus, the rate of fragmentation of intact cTnI into lower molecular weight fragments depended upon the cause of death.

In case of Control group the major bands of size 25, 23, 22, 21, 16, 14 and 7.1 kDa were observed at 0, 6, 12, 24, 48, 72 and 96 hrs respectively, Fig. 3.5.
Fig. 3.5. Western blot analysis of cTnI degradation in myocardium in Control subject. (Lane 1: Standard, Lane 2: 0 hrs, Lane 3: 6 hrs, Lane 4: 12 hrs, Lane 5: 24 hrs, Lane 6: 48 hrs, Lane 7: 72 hrs, Lane 8: 96 hrs)

In case of Acebutolol group the major bands of size 25, 15, 13, 12, 9, 7 and 7 kDa were observed at 0, 6, 12, 24, 48, 72 and 96 hrs respectively, Fig. 3.6.

Fig. 3.6. Western blot analysis of cTnI degradation in myocardium after death due to Acebutolol poisoning. (Lane 1: Standard, Lane 2: 0 hrs, Lane 3: 6 hrs, Lane 4: 12 hrs, Lane 5: 24 hrs, Lane 6: 48 hrs, Lane 7: 72 hrs, Lane 8: 96 hrs)
In case of Asphyxia group the major bands were of size 25, 21, 19, 17, 15, 12 and 7 kDa were observed at 0, 6, 12, 24, 48, 72 and 96 hrs respectively. Fig. 3.7.

Fig. 3.7. Western blot analysis of cTnI degradation in myocardium after death due to Asphyxia. (Lane 1: Standard, Lane 2: 0 hrs, Lane 3: 6 hrs, Lane 4: 12 hrs, Lane 5: 24 hrs, Lane 6: 48 hrs, Lane 7: 72 hrs, Lane 8: 96 hrs)

The data clearly established that the degradation pattern of cTnI is different for all the three groups- Control, Acebutolol and Asphyxia. In case of Acebutolol the degradation of cTnI into its molecular fragments is at a much faster rate when compared to the Control and Asphyxia groups, Fig. 3.8. The difference in degradation of cTnI is because Acebutolol is highly cardiotoxic, and its effect on cardiac tissues is high and immediate. The myocardial damage is maximum in case of Acebutolol poisoning and then in case of Asphyxia and then in Control group. The degradation of cTnI is dependent upon the severity of myocardial damage at the time of death.
The two groups were compared with the control group. It was observed that in case of death due to Acebutolol poisoning the rate at which intact cTnI fragmented into lower molecular weight fragments was high as compared to that of control and asphyxial group, Fig.3.2.

A one-way ANOVA was used to test for preference differences among the three groups. The null hypothesis was that no difference exists between the population. The molecular weights differed significantly across the three sizes, $F(2, 12) = 6.623334$, $p = 0.011531$, $F$-critical = 3.885294, Table.3.3. The F value is greater than the F critical value and thus the null hypothesis ($H_0$) is rejected.

The data was chosen from 6 hrs to 72 hrs. At 0 and 96 hours, the molecular weights of protein of different groups are the same, hence they are not included in the ANOVA test.

**Fig.3.8. Plot of cTnI degradation into its lower molecular weight fragments with time in Control, Cardiotoxicity (Acebutolol) and Asphyxia Groups**
Table 3.2. Comparison of Decrease in Molecular Weights of cTnI with respect to Time in Various Groups of Study

<table>
<thead>
<tr>
<th>Hours</th>
<th>Control</th>
<th>Acebutolol</th>
<th>Asphyxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>23</td>
<td>15.6</td>
<td>21.5</td>
</tr>
<tr>
<td>12</td>
<td>22.1</td>
<td>13.8</td>
<td>19</td>
</tr>
<tr>
<td>24</td>
<td>21.4</td>
<td>12.2</td>
<td>17.2</td>
</tr>
<tr>
<td>48</td>
<td>16.8</td>
<td>9.6</td>
<td>15.6</td>
</tr>
<tr>
<td>72</td>
<td>14.6</td>
<td>7</td>
<td>12.2</td>
</tr>
<tr>
<td>96</td>
<td>7.1</td>
<td>6.6</td>
<td>7.1</td>
</tr>
</tbody>
</table>
Table 3.3. Statistical Analysis of Change in Molecular Weights with Respect to Postmortem Interval in the three groups of Study

<table>
<thead>
<tr>
<th>Groups</th>
<th>Count</th>
<th>Sum</th>
<th>Average</th>
<th>Variance</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>130</td>
<td>18.5714285</td>
<td>38.6490</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Acebutolol</td>
<td>7</td>
<td>89.8</td>
<td>12.8285714</td>
<td>40.0590</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Asphyxia</td>
<td>7</td>
<td>117.6</td>
<td>16.8</td>
<td>35.17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ANOVA

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>121.078095</td>
<td>2</td>
<td>60.5390476</td>
<td>1.59483</td>
<td>0.23032</td>
<td>3.55455</td>
</tr>
<tr>
<td>Within Groups</td>
<td>683.268571</td>
<td>4</td>
<td>37.9593650</td>
<td>8</td>
<td></td>
<td>3.55455</td>
</tr>
<tr>
<td>Total</td>
<td>804.346666</td>
<td>7</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.5 Conclusion

The results of the present study clearly established that the degradation pattern of cardiac troponin I can be used for determining postmortem interval. The rate of degradation of cTnI is dependent upon the cause of death and can be used for establishing the cause of death. It was further established that the degradation of intact cTnI into smaller molecular fragments is also dependent upon temperature.

In order to use the measurement of cTnI fragmentation for the determination of the postmortem interval, further investigations in terms of real human sample cases is necessary to understand more about the cTnI degradation pattern in humans.

Cardiac troponin has a vast potential in forensic science and can be used for determining postmortem interval and also establish that whether the death was due to cardiac failure or not.
3.6 References


16. Singh OG, Gupta BD, Violent mechanical asphyxia deaths in homicide-a retrospective study of 5 years, J. of FORENSIC MEDICINE AND TOXICOLOGY


