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EILEEN MERCEDES. PARE

University of Windsor

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NL-339 (r. 82/08)

Canada
MORPHINE LEVELS IN BRAIN TISSUE
OF HEROIN ADDICTS

by

Eileen Mercedes Paré

A Dissertation
Submitted to the Faculty of Graduate Studies
through the Department of Chemistry
in Partial Fulfillment
of the Requirements for the Degree
of Doctor of Philosophy at the
University of Windsor

Windsor, Ontario, Canada
1982
ABSTRACT

MORPHINE LEVELS IN BRAIN TISSUE OF HEROIN ADDICTS

by

Eileen Mercedes Paré

In this study a relatively simple and efficient extraction and derivatization procedure has been used to extract a metabolite of heroin, morphine, from brain tissue. The bis(0-trimethylsilyl)morphine derivative formed is analyzed by gas chromatography using a 3% OV-17 column and a flame ionization detector. With the procedure as described it is possible to determine as little as 0.13 μg of morphine per gram of brain tissue.

The concentration of morphine in the blood of heroin addicts who die as a result of their addiction is often no higher than the level of those who do not die as attested to by the level of morphine found in the blood of homicide victims. It has been suggested that death is a result of rapid metabolism and distribution of the drug to organs where the concentration is fatal.

Since most of the opiate receptors are located in the brain and it is the site where most, if not all of the effects of the drug originate, the concentration of morphine in post-mortem samples of various areas of the brain have been determined and related to blood concentration.

The results from the analysis of 21 cases are presented.
When the correlation coefficients between the morphine concentrations in blood and in each of the brain sections analyzed were tested against the null hypothesis that the population correlation was zero, the correlation was significant at the 5 percent level between blood morphine concentrations in the blood and in the brain stem. The correlation was significant at the 1 percent level between morphine concentrations in the blood and in the thalamus.

The conclusion reached in this study is that the level of morphine in the blood can be used to predict the level in the brain, the best correlation being between the blood and the thalamus.
DEDICATION

to

Mark J. and Eileen C. Paré

whose love and faith have made life a happy experience
ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Dr. R. J. Thibert for his support and encouragement during the course of this work and throughout my program at the University of Windsor. I gratefully acknowledge the help of Dr. J. R. Monforte in procuring the specimen for this work and for his direction and advice during the project.

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TABLE OF CONTENTS

ABSTRACT .......... 11
DEDICATION .......... iv
ACKNOWLEDGEMENTS ... v
LIST OF FIGURES ... ix
LIST OF TABLES ... x
LIST OF ABBREVIATIONS ... xi

CHAPTER

I. INTRODUCTION ... 1
   A. General Information ... 1
   B. Heroin Metabolism in Man ... 4
   C. Postmortem Distribution and Concentration of Morphine ... 18
   D. Other Drugs ... 20
   E. The Brain and Opiate Receptors ... 22
   F. Analytical Methodology for Morphine ... 35
   G. The Study ... 50

II. EXPERIMENTAL ... 52
   A. Apparatus ... 52
   B. Materials ... 53
      1. Chemicals and Reagents ... 53
      2. Samples ... 54
   C. Method ... 54
      1. Analytical Studies of Aqueous Standards ... 54
      2. Recovery Study ... 55
      3. Analysis of Biological Samples ... 56

III. RESULTS AND DISCUSSION ... 58
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Analytical Studies of Aqueous Standards</td>
<td>58</td>
</tr>
<tr>
<td>B. Recovery Study</td>
<td>61</td>
</tr>
<tr>
<td>C. Analysis of Biological Samples</td>
<td>68</td>
</tr>
<tr>
<td>D. Case Histories</td>
<td>81</td>
</tr>
<tr>
<td>IV. SUMMARY AND CONCLUSIONS</td>
<td>86</td>
</tr>
<tr>
<td>A. Analytical Studies of Aqueous Standards</td>
<td>86</td>
</tr>
<tr>
<td>B. Recovery Study</td>
<td>87</td>
</tr>
<tr>
<td>C. Analysis of Biological Samples</td>
<td>87</td>
</tr>
<tr>
<td>D. Case Histories</td>
<td>89</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>92</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>95</td>
</tr>
<tr>
<td>VITA AUCTORIS</td>
<td>102</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>TITLE</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Morphine</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Metabolic Pathway of Heroin.</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Cumulative Urinary Excretion of Heroin and Metabolites</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>Model of Opiate Receptor</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>Opiate Drugs</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>Space-Filling Molecular Models</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>Standard Curve: Morphine Extracted from Water Solution</td>
<td>65</td>
</tr>
<tr>
<td>8</td>
<td>Representative Chromatograms</td>
<td>67</td>
</tr>
<tr>
<td>9</td>
<td>Calibration Curve Using Methanol Standards</td>
<td>71</td>
</tr>
<tr>
<td>10</td>
<td>Composite Curve: Brain Control Standards</td>
<td>76</td>
</tr>
<tr>
<td>11</td>
<td>Comparison of Morphine Concentration in Brain and Blood</td>
<td>80</td>
</tr>
</tbody>
</table>
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Urinary Excretion of Heroin Metabolites</td>
<td>.15</td>
</tr>
<tr>
<td>II Analysis of Morphine Extracted from Water Solution: Within-Day Variation</td>
<td>.62</td>
</tr>
<tr>
<td>III Analysis of Morphine Extracted from Water Solution: Between-Day Variation</td>
<td>.63</td>
</tr>
<tr>
<td>IV Recovery of Morphine</td>
<td>.69</td>
</tr>
<tr>
<td>V Analysis of Brain Samples</td>
<td>.77</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSTFA</td>
<td>N, O-bis(trimethylsilyl) acetamide</td>
</tr>
<tr>
<td>[D-Ala², D-Leu⁵]-enkephalin</td>
<td>[Leu]-enkephalin with the second and fifth amino acids being D rather than L isomer</td>
</tr>
<tr>
<td>ECD</td>
<td>Electron-capture detector</td>
</tr>
<tr>
<td>EMIT</td>
<td>Enzyme-multiplied immunoassay technique</td>
</tr>
<tr>
<td>ESR</td>
<td>Electron spin resonance</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionization detector</td>
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<tr>
<td>FRAT</td>
<td>Free-radical assay technique</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
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<tr>
<td>GC-MS</td>
<td>Gas chromatography - mass spectrometry</td>
</tr>
<tr>
<td>HI</td>
<td>Hemagglutination inhibition</td>
</tr>
<tr>
<td>HMDS</td>
<td>Hexamethylenebisilazane</td>
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<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<tr>
<td>I.V.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>kPa</td>
<td>Kilopascal</td>
</tr>
<tr>
<td>[Leu]-enkephalin</td>
<td>H₂N-tyrosine-glycine-glycine-phenylalanine-leucine - COOH, all L isomers</td>
</tr>
<tr>
<td>3-MAM</td>
<td>3-Monoacetylmorphine</td>
</tr>
<tr>
<td>6-MAM</td>
<td>6-Monoacetylmorphine</td>
</tr>
<tr>
<td>[Met]-enkephalin</td>
<td>H₂N-tyrosine-glycine-glycine-phenylalanine-méthionine-COOH, all L isomers</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
</tr>
<tr>
<td>TMCS</td>
<td>Trimethylchlorosilane</td>
</tr>
</tbody>
</table>
Tri-Sil Z
TSIM
Trimethylsilylimidazole in dry pyridine
Trimethylsilylimidazole
CHAPTER I
INTRODUCTION

A. General Information

Written reference to the use of poppy juice is found in the writings of Theophrastus in the third century B.C. but it is thought that the Sumerians (ca. 4000 B.C.) knew of the psychological effects of opium.

Morphine, the component of opium with the most marked analgesic activity, was isolated in 1805 and named after Morpheus, the Greek god of dreams (See Fig. 1). A pentacyclic alkaloid, natural morphine is levorotatory; the d-isomer has no significant pharmacological activity. Heroin (3, 6-diacetylmorphine) was introduced in 1898. It was a better analgesic and was originally thought to be nonaddictive. In addition to being an analgesic, heroin gives rise to a sense of euphoria and less desirable side-effects which include constipation, respiratory depression, nausea, hypotension and release of antidiuretic hormone. It has been found to produce physical dependence and tolerance more rapidly than morphine (1).

By 1974 the U.S. Bureau of Narcotics estimated that there were over 400,000 heroin addicts in the United States (2). In 1975 there were 341 narcotic addiction deaths recorded in Wayne County, Michigan (3).

The increasing abuse of narcotics has become a major problem for society and has led to a search for new methods for the study of addiction in man. Sensitive analytical methods have been developed for chemical and pharmacological
FIGURE 1
MORPHINE

(7,8-Didehydro-4, 5-epoxy-17-methyl-morphinan-3,6-diol)

Legend
Heroin, 3,6-diacetylmorphine, is rapidly hydrolyzed in the body to 6-monacetyl morphine and morphine.
FIGURE 1

MORPHINE

(7,8-Didehydro-4,5-epoxy-17-methyl-morphinan-3,6-diol)
research in this area (1,4).

B. Heroin Metabolism in Man

The rate of absorption, distribution, metabolism and excretion of heroin depends on the way in which it is administered. Heroin is usually administered parenterally, most addicts using intravenous injection.

The onset of pharmacologic effects of heroin is rapid following intravenous administration of the drug. Heroin is a more nonpolar molecule than morphine and has higher lipid and membrane solubility; thus there is rapid absorption from the bloodstream and passage through the blood-brain barrier. Because it has a half-life in blood of less than 20 min it would seem that except for the initial transient effect, known to addicts as a "rush", the pharmacological effects of heroin are mediated by 6-monoacetylmorphine (6-MAM) and morphine (1,2).

The metabolism of heroin was reviewed in 1975 (2). Heroin is hydrolyzed by an arylesterase preferentially at the 0\textsuperscript{3}-acetyl group rather than the 0\textsuperscript{6} site to form 6-MAM (5). The 0\textsuperscript{6}-acetyl group appears to be hydrolyzed by tributyrinase (6). In vitro studies by Way et al. showed that deacetylation of heroin to 6-MAM and morphine occurred in homogenates of human brain, kidney, liver and blood (7). Liver tissue exhibited the greatest hydrolytic activity and brain tissue the least. Morphine itself was not found to hydrolyze.

In vitro studies by Nakamura and Ukita with human blood indicated that heroin hydrolyzed rapidly, being undetected
after one h of incubation (8). It was hydrolyzed to 6-MAM which was stable in blood up to seven h - their final data point. They detected only a small amount of morphine and no 3-MAM. 6-MAM is then implicated as an important mediator of the pharmacologic activity of heroin. However, Garrett and Gurkan maintain that much of the data reported on the metabolism of heroin in plasma or biological fluid is suspect because of its rapid in vitro hydrolysis in aqueous solutions and biological fluids (9). They suggest the use of tetraethyl pyrophosphate as an inhibitor of the enzymatic hydrolysis of heroin in plasma prior to extraction under conditions where spontaneous hydrolysis would be minimized. As a result of its rapid hydrolysis, pharmacokinetic studies on heroin administration have been based on the half-lives of the urinary excretion of the major heroin metabolites.

6-MAM probably represents the major opiate leaving the bloodstream after heroin administration. 6-MAM is converted to morphine in the liver. Morphine then undergoes synthetic reactions as a means of detoxification and excretion. The predominant synthetic reactions are glucuronide formation and 3-ethereal sulfate formation. Both processes are catalyzed by well-defined enzyme systems in the endoplasmic reticulum of hepatocytes, placenta, brain, kidney and intestine. Quantitatively, the most important morphine metabolite formed is morphine-3-glucuronide. In one study of a 35-h pooled urine sample, morphine-3-glucurohicide accounted for 45% of the administered dose while morphine-6-glucuronide
was estimated to account for 0.3%. It has been reported that morphine-6-glucuronide is more active as an analgesic than morphine sulfate over a longer period of time (10). Morphine-6-glucuronide and morphine-3,6-diglucuronide have both been identified as morphine metabolites in the urine of morphine-dependent subjects (11-13). The amount of these metabolites was estimated to be about 1% that of morphine-3-glucuronide. It has been suggested that morphine-6-glucuronide could result from enzymatic conjugation in vivo or hydrolysis of morphine-3,6-diglucuronide either in vivo or during analysis, since the glucuronide conjugate at the 3-position hydrolyzes more easily than that at the 6-position (13). Between 5 and 10% of an administrated dose of morphine administered to drug dependent subjects was also recovered as morphine-3-etheral sulfate from 24-h pooled urine samples (14).

Normorphine formed by the oxidative biotransformation of morphine has been identified in the urine of patients given morphine sulfate intravenously during surgery and also in the urine of ex-addicts given a single intravenous dose of heroin hydrochloride (15, 16). In the latter case normorphine accounted for 4% of the administered dose. When Yeh analyzed, by gas chromatography, the 24-h urine samples of four morphine-dependent subjects receiving subcutaneous injections of morphine sulfate he found the mean daily percent excretion of the administered dose to be: 10% free morphine; 74% total morphine, free plus conjugated; 1% free
normorphine; and 4% total normorphine (17). The excretion of total drug was linearly related to the volume of the daily urine output. In some of the earlier studies neither free nor conjugated normorphine was reported. This may have been due to the poor solubility and extractability of normorphine from aqueous solution into water-immiscible organic solvents, the pH dependency of the extractability, and/or its instability in both acidic and especially alkaline aqueous solutions. Studies in vitro and in vivo in laboratory animals indicate that N-demethylation is decreased by chronic administration (17). The amount of free normorphine in urine increased after incubation with a-glucuronidase, suggesting that some of the normorphine conjugate was glucuronide, but was only about one-third of that liberated after acid hydrolysis, suggesting that a large portion of normorphine conjugate may not be glucuronide. Since N-demethylation reactions occur in man, the reconversion of normorphine to morphine may also take place.

Morphine-N-oxide has been found in the urine of cancer patients after administration of morphine sulfate and amphetamine (18). Although morphine-N-oxide has also been observed in guinea pigs it is thought to be an alternative oxidative metabolic product in man, probably induced by amphetamine.

Boerner and Abbott have identified codeine, both conjugated and free, in the urine of heroin addicts, two cardiac by-pass patients treated with morphine sulfate and one cancer patient (19). It has also been found by various laboratories
during urine drug screens (2). However, Yeş was unable to identify codeine or norcodeine as metabolites in his research on opiate metabolites in urine (11-14, 16, 17). It has been suggested that the reason for this discrepancy may be due to a difference in liver function between the subjects of the study (2). Addicts are often using more than one drug, one or more of which can induce liver microsome activity. Hepatitis is not uncommon in heroin addicts. The cardiac by-pass patients may have had abnormal hepatic enzyme activity resulting from chronic passive liver congestion. This last group was also receiving a number of other drugs which could change hepatic enzyme function.

Several additional metabolites, including morphine-N-oxide and/or α- and β-dihydromorphines, were identified in guinea pigs, rats and rabbits but not in man (12). However, the human urine analyzed had been refrigerated for 3-4 years prior to analysis. A possible heroin metabolic pathway in man is illustrated in Fig. 2.

The kinetics of the urinary excretion of heroin and its metabolites in humans has been investigated (16). Morphine can be identified in urine within 6 min following infusion of heroin. A single intravenous 10 mg/70 kg body weight dose of heroin hydrochloride was given to ten healthy postaddicts. Urine was collected before and at intervals of 1, 2, 6, 8, 16 and 24 h after drug administration. Free morphine, 6-acetylmorphine, free normorphine, morphine-3-glucuronide, morphine-6-glucuronide, 6-acetylmorphine-3-glucuronide and normorphine
FIGURE 2
METABOLIC PATHWAY OF HEROIN

Legend
A possible heroin metabolic pathway in man is illustrated. The pathway was taken from Boerner et al. (2). The inclusion of codeine in the pathway is highly controversial. Yeh has provided evidence (Yeh, S. (1974) Experientia 15, 264) that in man any codeine present in the urine is a result of the presence of acetylcodene formed as a byproduct in the manufacture of heroin or as a result of the presence of codeine as a contaminant in morphine sulfate. He also points out that in the GC analysis, if derivatization is by on-column acetylation, monoacetylmorphine and acetylcodene have similar retention times and cannot be resolved on either 3% OV-17 or 3% SE-30 columns at various temperatures. There is the possibility then of mistaking one compound for the other or believing the concentration of acetylcodene to be higher than that expected from contamination in illicit heroin.

N-Demethylation is the major metabolic pathway for cats and also for Gunn rats, which have hereditary defects in glucuronide formation.
FIGURE 2
METABOLIC PATHWAY OF HEROIN

HEROIN

6-MONOACETYL MORPHINE

MORPHINE

Continued on next page
glucuronide were found as metabolites. All of the excreted heroin, 74% of the excreted free 6-acetylmorphine, 56% of the excreted free morphine and 35% of the excreted total normorphine (free plus conjugated) were found in the first 2-h urine samples. Of the sum of conjugates (morphine plus 6-acetylmorphine) excreted in the 24-h urine, 25% appeared in the first 2 h and 47% in the first 4 h. Approximately 50% of the administered heroin was excreted as the sum of the conjugates (morphine plus 6-acetylmorphine) in the 24-h urine. Total normorphine and 6-acetylmorphine were not detected in the samples collected after 8 h. The cumulative excretion curves of heroin and its metabolites in the urine are shown in Fig. 3. Table I summarizes the mean percentages of the administered dose found in the urine as heroin and its metabolites in this study. The half-lives of the urinary excretion of the heroin metabolites were obtained by plotting the logarithms of the rates of excretion of the metabolite vs the midpoint of the collection periods. Linear curves were obtained for the early collection periods from the regression equation calculated by the least squares method. The half-lives were then calculated according to the first order kinetic equation. The regression line of the sum of conjugates (morphine plus 6-acetylmorphine) was taken from data points between 3 and 14 h. The rate of excretion of conjugates in the first 2 h was usually less than that in 2-to 4-h urine. This is probably due to the time lag of biosynthesis of the conjugates. Beyond 12 h the rate of elimination of the sum
FIGURE 3
CUMULATIVE URINARY EXCRETION OF HEROIN AND METABOLITES

Legend
Cumulative urinary excretion of heroin and its metabolites for 24 hours after i.v. administration of 10mg/70 kg of heroin HCl to 10 subjects. Samples were taken every two hours.

Mean ± S.E.

Data taken from Yeh, S. et al. (16).
FIGURE 3
CUMULATIVE URINARY EXCRETION OF HEROIN AND METABOLITES

% of Administered Dose

Total Morphine

Sum of Conjugates (Morphine plus Acetylmorphine)

Free Morphine

Total Normorphine

6-Acetylmorphine

Heroin

HOURS

0 2 4 6 8 10 12 14 16 18 20 22 24
TABLE I

URINARY EXCRETION OF HEROIN METABOLITES

<table>
<thead>
<tr>
<th>Metabolites identified</th>
<th>Study 1&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Study 2&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>aMean percentage of administered dose (+S.E.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Study 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Study 2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heroin</td>
<td>—</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>Free morphine</td>
<td>7.7±0.9</td>
<td>7.2±0.7</td>
</tr>
<tr>
<td>6-Acetylmorphine</td>
<td>0.9±0.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td>Total morphine (free + conjugated)</td>
<td>62.7±3.2</td>
<td>60.5±2.8</td>
</tr>
<tr>
<td>Total normorphine (free + conjugated)</td>
<td>4.8±0.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.8±0.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ten subjects were used in each study. Each subject received 10 mg/70 kg i.v. of heroin hydrochloride.

<sup>b</sup>Study 1 Urine collected every 8 hours and ad libitum for 1 week.

<sup>c</sup>Study 2 Urine collected every 2 hours in the first 8 hours, then less frequently for 24 hours.

<sup>d</sup>Was not detected.

<sup>e</sup>Only 3 of 10 samples showed 6-acetylmorphine.

The data for this table were taken from Yeh, S. et al. (16).
of conjugates appeared to increase. This multiphasic pattern has been reported by others (2,20). Spector and Vesell reported a similar disappearance curve from serum (21). They used radio-immunoassay rather than gas-liquid chromatography or gas chromatography-mass spectrometry to analyze serum samples from ten subjects who received 10 mg/70 kg of morphine sulfate intravenously. Their method measured morphine and some of its metabolites, higher concentrations being required for the glucuronide and the demethylated metabolites. In the first six h they determined the morphine half-life to be from 1.9 to 3.1 h. Afterwards the disappearance was very slow with a half-life of from 10 to 44 h. The rapid phase may represent distribution of the opiate between blood and tissue, followed by metabolism and excretion. The prolonged presence of morphine and its conjugates could result from the reabsorption of morphine from the intestines after hydrolysis of the conjugated morphine released by the gallbladder and/or the prolonged retention of protein-bound drug.

In an in vitro study Ryan et al. found that 40% of the addicts' sera exhibited significantly higher α-globulin binding of tritium labeled morphine than nonaddicts (22). There was only slight binding observed in nonaddicts. α-globulin levels were similar in the sera of both groups.

The recovery of total drug in the urine after administration of 10 mg of heroin hydrochloride per 70 kg ranged from 55 to 95%, the average being about 80%, with excretion linearly related to urine output (16).
Morphine and its metabolites are also excreted via bile in the feces (23). Four non-tolerant volunteers excreted 6.7 to 10.4% of the administered dose of ^14^C-labelled morphine sulfate in the feces within 72 h. The only addict studied excreted 0.1% of the radioactivity by way of the feces.

Gorodetzky and Kullberg analyzed the saliva and plasma of five volunteers for morphine after the injection of 10 mg of heroin (24). All of the samples were positive at levels <30 ng/mL 15 min after injection. The level in saliva declined after one h but was still detectable 12 h later in 20% of the specimens. Gorodetzky also found morphine in perspiration 1 to 8 h after the injection of 10 mg of heroin (17).

RIA has been used to determine morphine in the hair of heroin addicts (25, 26). Since hair grows at the rate of about 1 cm/month, the time course over which the drug has been taken can be determined by testing hair segments at different distances from the hair root.

In any study of the administration, distribution and metabolism of heroin in man it is important to keep in mind the biological variation between individuals. Of necessity the number of individuals studied is small. There seem to be obvious differences in the biotransformation and rate of excretion between addicts and nonaddicts. There also appear to be differences between the well and the ill. It has even been suggested that differences may exist between the sexes (27).
C. Postmortem Distribution and Concentration of Morphine

In a significant number of heroin fatalities the level of opiate found in the blood of addicts who die, apparently as a result of narcotic addiction, is below the minimum level generally accepted as fatal (3). In cases of drug overdose, the minimum lethal concentration of morphine has been reported as 0.2μg/mL of blood and 0.2-0.4μg/g of muscle (4). The levels are somewhat higher for addicts.

A study by Monforte indicated that the frequency of detecting morphine in the blood of homicide victims in Detroit, 88% of the cases, was about the same as that in narcotic addiction deaths, 83% of the cases (3). None of the homicide victims had a blood morphine concentration greater than 0.25μg/mL. The interesting fact is that in most of the narcotic addiction deaths (>75%) the blood morphine level was also less than 0.25μg/mL.

In one study of 129 heroin deaths only 61% gave a positive blood morphine level (28). In another study of 250 heroin fatalities 70% were positive for blood morphine. In the remaining cases no morphine was detected in the blood but was found in other tissues (29).

It has been suggested that the suddenness of many deaths was due to a shocklike reaction to excipient materials found in illicit narcotic preparations (30). Garriott and Sturner were unable to observe the typical anaphylactic response of an acute hypersensitivity in a study of 22 acute heroin fatalities (31). They supported the theory that intravenous
injections of lethal doses of the narcotic cause centrally induced respiratory depression or direct toxic effects on the smooth muscle of alveolar capillaries of the lung. Nakamura believes that in most cases it can be shown that the narcotic was rapidly distributed through the body to various organs where levels were lethal and it may now be unnecessary to blame excipients or hypersensitivity responses (29).

For an accurate interpretation of the toxicologic data in drug fatalities the time interval between the last administration of the drug and the resulting death is important. Because of the criminal nature of the act this information is often impossible to obtain.

Survival time has been correlated with blood levels by several groups (29,31-33). Addicts who survived less than 3 h had blood levels between 0.1 to 1.0μg/mL. In 28 cases where survival time ranged from 2 to 14 h morphine was detected in the blood of only 7 cases, the range being 0.1 to 0.8μg/mL (29).

As a result of the rapid metabolism of heroin and morphine it has been suggested that more information could be gained by analysis of samples other than blood.

Christopoulos and Kirch found that the relative concentrations of morphine in urine and bile correlate with the type of heroin poisoning (34). If the bile value is higher than the urine value, then death is acute. If the urine value is higher then death is due to chronic poisoning.

When liver samples were analyzed for morphine 94% of
the samples reported by five investigators were in the range of 0.1 to 10.0μg/g (4, 29, 34, 35). Felby et al. found that the blood to liver ratio was 1:5(4). They also observed that there was no correlation between morphine concentration in urine and that in blood. Robinson and Williams reported a blood to liver morphine ratio of 3:20 for heroin users (35). Nakamura found a ratio of 1:5 for 31 out of 35 heroin fatalities while in the other four cases the ratio was 1:30 (29).

A correlation between liver concentrations and estimated survival time has also been made. The average concentration for 47 cases whose survival time was less than 2 h was 1.5μg/g while those who survived from 3 to 15 h had an average concentration of 0.8μg/g (29). In addition the ratio of liver to kidney concentration went from 1:1.1 for survival time of approximately one h to about 1:1.5 for a survival time of 5 to 14 h.

D. Other Drugs

The interpretation and correlation of data obtained during postmortem examination is often complicated by the presence of more than one drug. In heroin fatalities alcohol, also a central nervous system depressant, is often present, e.g., in 32% of 252 cases in Los Angeles County, 47% of 129 cases in San Francisco and 40% of the cases in New York City (29). The synergistic effects of the two drugs is not completely understood. Monforte has observed that as the blood morphine concentration increases the quantity of ethanol that can be tolerated decreases (3). Jeffcoate has suggested that alcohol
stimulates the release of endogenous opioid peptides (36). His suggestion is based on the fact that naloxone prevented the impairment of psychomotor performance by alcohol as measured by the four-choice serial reaction time test. Miller and his associates have reported that ethanol and other aliphatic alcohols selectively inhibit the binding of enkephalin and its stable analogs at concentrations that have little or no effect on the binding of opiate alkaloids. Since aliphatic alcohols are known to increase the fluidity of cell membranes and cell fluidity can affect the normal function of some membrane-bound proteins, they suggest that δ-receptors are more strongly influenced by changes in membrane fluidity than μ-receptors (37).

Quinine is a common adulterant in illicit heroin, particularly in the east and midwestern United States (3,29,38). Cardiac arrhythmia has been associated with sudden death when heroin and quinine are combined (39). In a study of 341 narcotic addiction deaths in the Detroit area, Monforte found that in 74 cases where the only drugs present in the blood were morphine and quinine, only in a few cases did the level of quinine exceed the therapeutic range of 3 to 5 μg/mL (3). The lethal level is considered to be greater than 30μg/mL (3).

In postmortem tissues from heroin addicts, a wide range of drugs have been detected including amphetamine, methamphetamine, cocaine, desipramine, ethchlorvynol, salicylate, diazepam and chlorpromazine (29).
E. The Brain and Opiate Receptors

Although brain tissue may not be routinely analyzed in the toxicology laboratory, the brain is of particular interest because of the high concentration of opiate receptors localized in the limbic system, primarily in the amygdala, corpus striatum and the hypothalamus (40-42). Opiate receptors are also concentrated in the central gray matter of the brainstem and the central part of the thalamus along the paleospinothalamic pathway. Within the brain stem opiate receptors are highly localized in the solitary nuclei, which may account for the way in which opiates depress the cough reflex and reduce gastric secretion, and in the area postrema, the apparent site where opiates induce nausea and vomiting.

Drugs, hormones and neurotransmitters all produce their highly selective effects at very low concentrations. It is usually assumed that they act at specific receptor sites consisting of large molecules localized on the external surface of cells in the target organ. There is ample evidence that the opiates behave in like fashion.

The opiates can be divided into two groups: the agonists, those compounds like morphine and heroin which are analgesics and addictive; the antagonists, devoid of analgesic, addictive and other opiate agonist properties. In fact opiate antagonists are given to counteract the effects of an overdose of an agonist. Compounds have been prepared which have properties of both agonist and antagonist and it is from among such compounds
that an analgesic that is not addicting may be found. Agonists can be distinguished from antagonists in receptor binding assays. Low concentrations of sodium increase the binding of antagonists and decrease the binding of agonists.

Opiate agonists exhibit a high degree of steric and structural specificity. In a large number of morphine-like analgesics studied, it is always the levorotatory enantiomer that is active. Small changes in molecular structure frequently result in drastic changes in pharmacological potency. Substitution of the N-methyl group on morphine for an allyl group results in an antagonist, nalorphine, with few agonist properties. Experimental evidence seems to indicate that sodium has an allosteric effect on the conformation of the opiate receptor decreasing its affinity for agonists (43, 44). Figure 4 shows a model proposed by Snyder to explain the sodium effect (41). Figure 5 illustrates the similarities and differences in structure between agonists and antagonists. The benzene ring A and the nitrogen are universal and seem crucial in both receptor conformations. The agonist form of the receptor seems to have a binding site for ring F. Morphine is not a strong agonist because it lacks ring F and the F ring of methadone apparently cannot assume the proper orientation to be a strong agonist. The length and flexibility of the N side chain appears to determine whether an antagonist has any agonist properties. The presence of a hydroxyl group adjacent to the allyl group on naloxone
FIGURE 4
MODEL OF OPIATE RECEPTOR

Legend

It has been suggested that the receptor can exist in two different conformations. Opiate antagonists have a high affinity for the sodium-binding conformation while opiate agonists have a high affinity for the no-sodium conformation.

Model taken from Snyder (41).
FIGURE 4
MODEL OF OPIATE RECEPTOR

Mixed Agonist-Antagonist

Antagonist

Sodium

Opiate Receptor in No-Sodium Conformation

Agonist
FIGURE 5
OPIATE DRUGS

Legend
An examination of the structures shown here suggests possible relationships between the molecular structure and pharmacological activity. The benzene ring A and nitrogen are present in all compounds. The strongest agonists appear to have a binding site for ring F also. Since methadone is not a strong agonist the F ring must not have the correct orientation to ring A for optimum binding. In addition the side chain of agonists apparently binds to a specific site.
If the side chain is flexible and rotates freely, some of the molecules can assume the agonist orientation resulting in a mixed agonist-antagonist. If the side chain is rigid the compound is an antagonist.

Structures taken from Snyder (41).
FIGURE 5

OPIATE DRUGS

Phenazocine-strong agonist

Morphine-agonist

Methadone-agonist

Pentazocine-mixed agonist-antagonist

Nalorphine-antagonist with some agonist activity

Naloxone-pure antagonist
restricts its flexibility resulting in a pure antagonist.
The allyl group of nalorphine can apparently flex enough
to bind to the agonist conformation of the receptor giving
the compound some agonist activity.

In 1975 Hughes identified the first endogenous opiate,
an extract from pig brain (45,46). The active factor was
identified as two pentapeptides, H-Tyr-Gly-Gly-Phe-Met-OH
and H-Tyr-Gly-Gly-Phe,Leu-OH which were called methionine
[Met]- and leucine [Leu]-enkephalin, respectively (47).
Terenius and Wahlström isolated similar substances from rat
and calf brain (48). A number of other peptides with opioid
properties have been isolated from extracts of the hypothala-
mus and pituitary glands. The generic term endorphin has
been used for these compounds.

Comparing the conformation of [Met]-enkephalin to that
of the opiate agonists in Fig. 6, the benzene ring of tyro-
sine is in precisely the same orientation as the benzene
A ring and the second ring, of phenylalanine, corresponds
to the F ring found in strong agonists. Fluorescent dye has
been affixed to guinea pig antibodies to cattle brain
enkephalins and the antibodies used to determine the location
of enkephalin nerve terminals (49). The distribution was
comparable to that found for the opiate receptors by auto-
radiographic mapping (41). [Met]-Enkephalin and [Leu]-
enkephalin are rapidly metabolized in vivo and in vitro (50).
Many analogs of enkephalin have been synthesized and tested
for opiate activity and stability. Replacement of glycine
FIGURE 6

SPACE-FILLING MOLECULAR MODELS'

Legend
The models of morphine and methionine-enkephalin have
some structural features in common. The benzene ring A of
morphine is in exactly the same orientation as the benzene
ring of the amino acid tyrosine on the enkephalin peptide
chain. The amino acid phenylalanine is in the same position
as the benzene F ring found in those opiates which are strong
agonists.

Models taken from Snyder (41).
FIGURE 6

SPACE-FILLING MOLECULAR MODELS

Methionine Enkephalin

Tyrosine

Phenylalanine

Glycine

Glycine

Methionine

Morphine

Ring A
at position 2 by D-Ala and substitution of leucine at position 5 by D-Leu has yielded a stable and active compound \( [D-\text{Ala}^2, \text{D-Leu}^5] \)-enkephalin useful in receptor binding assays.

A number of experiments provide evidence that multiple opiate receptors exist in animals (50-52). For example, by using \(^{125}\text{I}\)-labeled \( [D-\text{Ala}^2, \text{D-Leu}^5] \)-enkephalin and \(^3\text{H}\)-labeled naloxone at very low concentration but high specific radioactivity in affinity binding assays it is possible to distinguish two distinct types of opiate binding sites in rat brain; one binds enkephalins with higher affinity than narcotics while the other binds narcotics with higher affinity. The type of opiate receptor that binds morphine-like compounds preferentially is called the \( \mu \)-receptor and that which is more sensitive to enkephalin, the \( \delta \)-receptor. In addition to these two types several others have been postulated. \( \kappa \)-Receptors are most sensitive to the agonists ketocyclazocine and ethylketocyclazocine. Two types of receptors sensitive to certain mixed agonist-antagonists such as nalorphine and N-allylnormetazocine have been proposed, \( \sigma_1 \), a high affinity receptor which responds to modest doses of these drugs producing hallucinogenic and dysphoric effects and \( \sigma_2 \), a low affinity \( \sigma \) receptor which responds to large doses producing mydriasis and delirium in dogs (53). The longer chain peptides, such as, \( \beta \)-endorphin, bind selectively to an opiate receptor in the vas deferens of the rat, the \( \epsilon \)-receptor (52). In addition to \( \mu \) and \( \delta \)-receptors
a third type receptor has been reported in rat brain (54). It is being called the benzomorphan binding site because most benzomorphan drugs in the chemical family of 6,7-benzomorphan-11-ol with bulky substitutions at the nitrogen atom exhibit relatively high affinity.

It has been suggested that the difference between \( \mu \)- and \( \delta \)-receptors may be in the distance between the binding regions corresponding to the two aromatic rings of the tyrosine and phenylalanine residues of the ligand, the distance being shorter in \( \mu \)-receptors than in \( \delta \)-receptors (53).

Light microscopic autoradiography has been used to differentiate between \( \mu \)- and \( \delta \)-receptors in the rat central nervous system (55). In the frontal cortex the \( \mu \)-receptors and \( \delta \)-receptors are in different layers. In the corpus striatum \( \mu \)-receptors are in high concentration in clusters and as a subcallosal streak while the \( \delta \)-receptors occur diffusely. In the hippocampus the \( \mu \)-receptors are concentrated in the pyramidal cell layer while low concentrations of \( \delta \)-receptors are evenly distributed. The olfactory tubercle, the nucleus accumbens and the amygdala have a relatively high concentration of \( \delta \)-receptors and very low levels of \( \mu \)-receptors. The thalamus and hypothalamus contain high concentrations of \( \mu \)-receptors but very few \( \delta \)-receptors. The \( \mu \)-receptors in the thalamus are especially concentrated in the dorsomedial nucleus of the thalamus. In the midbrain the inferior colliculi, the periaqueductal gray, the median raphe and the interpeduncular nucleus are enriched in \( \mu \)-
receptors. The periaqueductal gray and median raphe have very low levels of δ-receptors, while moderate concentrations occur in the inferior colliculi and interpeduncular nucleus. The pontine nuclei have a relatively high concentration of δ-receptors and few μ-receptors. Other caudal areas that display relatively high concentrations of both μ- and δ-receptors include the nucleus tractus solitarius, vagal fibers, the nucleus ambiguus and the substantia gelatinosa. Chang and Cuatrecasas note that high concentrations of μ-receptors are found in areas that are relevant to pain sensation (53). They suggest that μ-receptors may mediate opiate induced analgesia. Since many of the areas high in δ-receptors are parts of the limbic system which is associated with control of emotions and reward behavior, δ-receptors may mediate the euphoric and affective actions of opiates.

Zhang and Pasternak have a somewhat different concept of the multiplicity and function of opiate receptors (56). They conclude that all opiates and opioid peptides exert their analgesic effect at a single high affinity site which they call μ₁. They also identify a low affinity site, μ₂, which binds opiates preferentially and another low affinity site which binds enkephalins much more strongly than morphine corresponding to a δ-receptor. Further experimentation and discussion would appear necessary to resolve these apparent anomalies.

In attempting to correlate the concentration of morphine
in the brain with the location of opiate receptors there are several problems. Most of the work, especially on the location of the various types of opiate receptors, has been done on rat brains. However, there is evidence that the distribution of receptors in the rat brain resembles that in the human brain (40, 42, 57, 58). Goldstein et al.

incubated mouse brain homogenates with $^{3}H$-levorphanol in the presence of a large excess of unlabeled levorphanol or its inactive enantiomer dextrorphan (59). Only about 2% of the total bound was stereospecific. Even that binding which is stereospecific may not be bound to opiate receptors since Pasternak observed that opiates can bind stereospecifically to glass filters (41).

The concentration of morphine found in the brain is a function of the time between the last dose and death. When plasma levels were compared to brain levels in rats after a single i.v. injection of morphine, the brain concentration was greatest within 5 min of injection (60). The ratio of brain concentration to plasma rose from 0.18 at 5 min to 1.0 at 60 min where it remained until 120 min, finally declining to 0.4 at 4 h after injection. Mullis et al. determined the half-life of morphine in rat brain tissue to be biphasic with an initial half-life of approximately 2 h followed by a slower half-life of about 5 h between 4 and 48 h after a single subcutaneous injection of radiolabeled morphine sulfate (61). Radioactivity could still be detected, equivalent to 10-20 ng morphine/g, in
the brain three weeks after a single subcutaneous injection (62).

F. Analytical Methodology for Morphine

Because of the rapid metabolism of heroin, primarily to morphine and its conjugates, it is morphine that is analyzed in heroin fatalities.

The literature on the analysis of morphine is extensive. The methods of analysis have been reviewed(1,63). The primary methods currently in use include: thin-layer chromatography (TLC); gas chromatography (GC); spectrofluorometry; immunoassay; high-performance liquid chromatography (HPLC); and gas chromatography-mass spectrometry (GC-MS).

Thin-layer chromatography has been the most commonly used method in screening for morphine and other drugs of abuse (64-68). Technical improvements have been made in the solvent systems, plates and sprays. The method is rapid, sensitive and economical. Loh et al. describe a procedure in which a n-butanol extract of morphine in urine is reacted with dansyl chloride (69). Picogram quantities of the highly fluorescent morphine derivative formed are visible under ultraviolet light after development and drying of the polyamide plate (69).

A sensitive fluorometric method has been developed for morphine in plasma and brain (70). Morphine is extracted from the tissue, saturated with sodium bicarbonate using a 10% solution of n-butanol in chloroform. The morphine is then back extracted into hydrochloric acid. The pH of the acid solution is adjusted to 8.5 and the morphine oxidized to pseudomorphine with potassium ferricyanide. After ten min the sample is
excited at 250nm and the fluorescence measured at 440nm. The method can measure 0.1μg morphine per mL sample. The pseudomorphine is stable for about 20 min if a mixture of potassium ferricyanide and ferrocyanide with a molar ratio of 16:1 is used as the oxidant. Normorphine, 6-acetylmorphine, dihydro- morphine and n-allyl morphine yield identical fluorescent spectra. This procedure has been adapted to the automated analysis of morphine in urine using Technicon AutoAnalyzer equipment (71). Fluorescence has also been obtained without the use of potassium ferricyanide by treating a chloroform- isopropanol extract with concentrated H₂SO₄ followed by concentrated NH₄OH and autoclaving at 120°C for 15 minutes (72).

Various types of immunochemical techniques have been applied to the analysis of morphine. They include: radioimmunoassay (RIA), free-radical assay technique (FRAT), enzyme-multiplied immunoassay technique (EMIT) and hemagglutination inhibition (HI). The immunoassays are based on the competitive displacement of labeled morphine from an antibody-morphine complex by nonlabeled morphine from the sample. The extent of this displacement is then measured by an instrumental method appropriate to the assay. The proportion of the labeled morphine displaced is related to the amount of nonlabeled morphine present in the sample through the use of a standard curve. A major advantage of immunoassays is that the biological fluids to be analyzed can be tested directly without sample extraction or concentration, which significantly reduces analysis time.
A RIA for morphine was first reported by Spector and Parker (73). Morphine was converted to 3-0-carboxymethylmorphine by reaction of the free base with sodium-8-chloroacetate in absolute ethanol. The carboxymethylmorphine was coupled to bovine serum albumin (BSA) in aqueous solution in the presence of a water soluble carbodiimide to form the immunogen. The immunogen was buffered to pH 7.4, emulsified with Freund’s adjuvant and injected subcutaneously into a rabbit. Blood was collected from the rabbit about 6 weeks after a booster dose of the immunogen was administered. The antiserum (serum containing the antibodies) was separated and assayed for antigen binding capacity (74). Then fixed amounts of the antiserum and [3H]-dihydromorphine were incubated with samples of unlabeled morphine of known concentration. The unlabeled morphine, the antigen, competes with the radiolabeled antigen for the antibody sites in the antiserum. After incubation a saturated solution of ammonium sulfate was used to precipitate the antigen-antibody complex, the bound antigen. The precipitate was dissolved after thorough washing and the radioactivity measured in a liquid scintillation spectrometer. A standard curve was constructed of concentration of nonradioactive morphine vs % of inhibition of binding of [3H]-dihydromorphine. Samples containing unknown quantities of morphine were run and the concentration of morphine determined from the standard curve. Various modifications have been made since the original procedure appeared in the literature (1,67). Other than the usual hazard in RIA of working with radioactive material the principal problem
with all immunoassays is cross-reactions of the antiserum with compounds similar to morphine. Spector and Parker found that codeine was a better inhibitor of radiolabeled binding than morphine, which wasn't surprising since codeine has greater structural similarity to the immunizing carboxymethyl-morphine group than morphine does. Attempts have been made to reduce cross-reactions by using different haptons to produce the antiserum. Antibody to morphine-6-hemisuccinate conjugated to BSA and purified by affinity chromatography was coated on polystyrene tubes (75). Buffer, $[^3H]$-dihydromorphine and the samples were incubated in the tubes, after which aliquots of the supernatant were counted. The amount of bound radiolabeled antigen was determined by difference. The assay is reported to be sensitive to as little as 0.5 ng of morphine per mL of serum. Although cross reactivity to the major metabolite of morphine, morphine-3-glucuronide, is 55-fold less than with morphine, there is extensive cross reactivity with other opiates (76). To measure radioactivity a liquid scintillation spectrometer is required for $[^3H]$-or $[^14C]$-labeled antigens and a gamma scintillation spectrometer for $^{125I}$-labeled antigens.

The EMIT assay is a homogeneous enzyme immunoassay, homogeneous because no separation step is required. Morphine is conjugated to the enzyme lysozyme. When the enzyme-labeled morphine is bound to the antibodies in the antiserum the enzyme is rendered inactive. Free morphine in a sample competes with the enzyme-labeled morphine for the antibody
decreasing the antibody-induced inactivation of the enzyme. Lysozyme catalyzes the breakdown of bacterial cell wall. The sample, a bacterial suspension and the enzyme-labeled morphine are introduced into a spectrophotometer set at 436 nm: the absorbance at 10 and 50 sec is recorded and the difference between the two readings is proportional to the concentration of morphine in the sample. A standard curve must be prepared. The presence of endogenous lysozyme in the sample leads to false positives. A blank should be run for each positive result. The sensitivity of the EMIT assay for morphine is 0.5μg/mL sample. An EMIT assay for morphine in urine is commercially available in kit form from Syva.

The FRAT is also a homogeneous immunoassay. Morphine is spin-labeled with a paramagnetic label such as nitroxide. When the spin-labeled morphine is mixed with the antiserum and a sample which contains morphine there is a competition between the spin-labeled morphine and the free morphine for the limited number of antibody sites. Unbound nitroxide-labeled morphine gives an electron spin resonance (ESR) spectrum of three sharp lines while the bound spin-labeled drug gives a broad ESR spectrum. The peak intensities can be compared to those obtained with known concentrations or morphine. The FRAT opiate assay is reported to be sensitive to 0.5μg morphine/mL (67).

In the HI assay the presence of morphine in a test sample decreases the amount of agglutination of morphine-coated red blood cells by the antibodies in the antiserum.
The assay is reported sensitive to 20-30 ng/mL but is semi-quantitative (67).

High-performance liquid chromatography has been successfully used to analyze for morphine. Before analysis the drug must be extracted from the biological sample and concentrated. Jane and Taylor used fluorescent detection after injecting the sample and potassium ferricyanide onto the column at the same time (77). Dihydromorphine was used as an internal standard. Three fluorescent dimers were formed, pseudomorphine, dehydromorphine dimer and a mixed dimer of the two compounds. The compounds were separated on a 7-μm Partisil porous silica column using a solvent system of methanol-2N NH₄OH-1N NH₄NO₃ (30:20:10). The use of HPLC eliminates the interference of other drugs which might be present in the biological sample and undergo the oxidative coupling reaction forming fluorescent products.

Morphine extracted from blood samples, with nalorphine added as the internal standard, has been injected onto a reverse phase column containing octadecyl-silane bonded to silica and eluted with a mobile phase consisting of 85% methanol and 15% 0.01 M KH₂PO₄ buffer at 40°C (78). Using an electrochemical, or amperometric, detector, morphine was detected at concentrations of less than 1 ng/mL. The detector is composed of a low-volume flow cell, 3 electrodes and an electronic detection unit. The electrodes are a glassy carbon working electrode, a glassy carbon auxiliary electrode and a silver/silver chloride reference electrode. Maximum
sensitivity was obtained when a voltage of +0.9 V was applied to the oxidizing electrode (79). A reduction in elution time has been achieved by using a normal phase 5 µm cyano-propyl column with a mobile phase consisting of 75% acetonitrile and 25% 0.02 N KH₂PO₄ with electrochemical detection (79). HPLC with electrochemical detection does not require derivatization and is reported to have detection limits for morphine which exceed those of GC-MS or electron-capture gas chromatography (79).

The analytical technique most often used for the analysis of morphine in the past has been gas chromatography or gas chromatography followed by mass spectroscopy. For the analysis of biological samples it is desirable to be able to detect morphine in the nanogram range with sensitivity and specificity. Methods based on radioimmunoassay, spectrofluorometry and gas-liquid chromatography are sufficiently sensitive but only gas chromatography is specific enough. GC coupled with mass spectrometry offers the best method available for the unequivocal identification of morphine in biological samples. It can be used to check the results of less sensitive and specific assays as well as for positive confirmation of structure.

Because of its usually low concentration, amphoteric nature and metabolism to a water soluble product, primarily morphine-3-glucuronide, morphine is relatively difficult to analyze in biological samples. To determine total morphine the glucuronide must first be hydrolyzed to free morphine
for extraction into an organic solvent. Both acid hydrolysis and enzymatic cleavage have been used (11,17). Enzymatic hydrolysis has generally proven to be less efficient than acid hydrolysis. A recovery of about 80% of radiolabeled morphine was achieved when biological samples, obtained from dogs injected with the drug, were incubated with β-glucuronidase/aryl sulfatase for 40 hours at 37°C. Critical parameters included choice of the buffer system, pH, temperature and enzyme concentration. In acid hydrolysis the effects of temperature and the amount of concentrated hydrochloric acid added were found to be interdependent. A recovery of more than 90% has been obtained when bile and urine samples were autoclaved at 103 kPa and 122°C for 45 min in solutions containing 5% v/v HCl. The same recovery was obtained when samples containing 20% v/v HCl were heated on a steam cone overnight at 75°C. Hydrolysis conditions vary widely in the literature. Complete hydrolysis of morphine-3-glucuronide in 30 min at 100°C by 4N HCl has been reported (81). In the GC/MS assay recommended by the National Institute on Drug Abuse equal volumes of specimen and 6N HCl are heated in a boiling water bath for 1 h (1).

After hydrolysis the sample must be neutralized, i.e., by the slow addition of dilute NaOH. Then the pH of the solution must be adjusted and the morphine extracted from its biological matrix to prevent contamination of the GC column. Because of its phenolic hydroxide ($pK_a$ 9.85) and its tertiary amine structure the pH of the aqueous phase should be
maintained slightly below the $pK_a$ of the phenol group with an appropriate buffer, the usual range being 8.5 to 10.0. The isoelectric point of morphine is 8.9. Many buffers have been reported in the literature, the most common being bicarbonate, carbonate or borate. The addition of salt is often used to aid in increasing the extraction. Sodium bicarbonate and ammonium carbonate are said to be preferred (80). In an investigation of solvents for the extraction chloroform was found to be the best single solvent, 25% better than methylene chloride (82). Isobutanol added to the chloroform (1:9) significantly improved the extraction. The addition of an alcohol not only increases the partition ratio but prevents adsorption to glassware (83,84). Predmore et al. looked at the extraction efficiency of eight solvent systems and concluded that chloroform/isopropanol in the ratio of 4:1 seems to be the solvent of choice because it removed the most morphine in a single extraction and was readily volatile, an important consideration since the solvent is evaporated eventually (80). They also recommend a solvent to sample ratio of at least three. Following the extraction the organic layer may be washed with a buffer solution to increase the removal of contaminants. The morphine is back extracted into a dilute aqueous solution of a mineral acid, usually HCl, for further purification. The aqueous solution is then made basic and the morphine extracted again into the same or another organic solvent system. Solvents which have been used in the extraction
include: chloroform with isopropanol or isobutanol in ratios of 9:1 to 3:1 (4,17,35,82,85,86): dichlorehane and isobutanol, ratio 3:1 (36), ethyl acetate and isopropanol, ratio 9:1 (87); toluene and n-butanol, ratio 9:1 (84) and toluene, heptane and isoamyl alcohol, ratio 7:2:1 (1).

Ikekawa et al. have isolated morphine from urine by charcoal absorption followed by solvent extraction and GC analysis (88). A styrene-divinylbenzene, neutral copolymer (XAD-2) has also been recommended for this purpose (67).

Initially gas chromatography was used for the detection of morphine as the free base in milligram quantities after extraction from opium preparations or street samples. Because of the polarity associated with phenolic alkaloids such as morphine, there is nonlinear adsorption of the drug onto the chromatographic column (86,87,89). This problem can be partly overcome by deactivation of the column and it can be eliminated if morphine is converted into a volatile non-polar derivative (89,90).

Anders and Mannerling described the on-column esterification of morphine in which acetic anhydride or propionic anhydride was injected onto the column within 5 seconds of the injection of the morphine extract (91). They also attempted to form the trimethylsilyl ether of morphine using a mixture of hexamethyldisilazane and trimethylchlorosilane but only obtained a 50% conversion. Rasmussen succeeded in the on-column derivatization of morphine to form bis-(0-trimethylsilyl) morphine by injecting trimethylsilylimidazole
and the sample at the same time (90). Verification of the compound was made by GC-MS.

Most recent work has concentrated on pre-chromatographic derivatization of morphine. Acetic anhydride has been used to convert the free base to diacetylmorphine for the detection of submicrogram quantities of morphine in urine and tissue samples (92). Wallace et al. described the use of trifluoroacetic anhydride for derivatization (82). Under their experimental conditions the principal product was the disubstituted derivative but mass spectra detected some monosubstituted derivative. However, they reported that the method was capable of detecting morphine levels down to 25 ng/mL in 2 mL of blood plasma with acceptable precision. Yeh had reported that the trifluoroacetic anhydride derivative was unstable (93). Dahlstrom and Paalzow used pentafluoropropionic anhydride for derivatization and reported that 100 pg of morphine could be determined in 30 mg of rat brain tissue (94). Heptfluorobutyric anhydride has also been used to detect morphine in urine at a concentration of 0.1μL/L (95).

Thenot and Haegele believe that the most satisfactory derivative of morphine for analytical purposes is the bis(trimethylsilyl) ether (83). The ability to prepare silyl ethers has greatly facilitated the conversion of high molecular weight polyfunctional substances into derivatives that are thermally stable and sufficiently volatile to be vaporized at moderate temperatures. Replacement of one of the methyl
groups in the trimethyl ethers with a halocarbon group enables derivatives to be prepared which can be selectively detected at trace levels with an electron-capture detector. The electron-capture detector is one of the most sensitive GC detectors available but is limited to those molecules which have the ability to accept a negative charge such as halogenated hydrocarbons, conjugated carbonyls and nitrates (81). The trimethylsilyl ethers have been prepared using various reagents including: hexamethyldisilazane (HMDS) together with trimethylchlorosilane (TMCS) which acts both as an acid catalyst and silyl donor (89); N,O-bis(trimethylsilyl) acetamide (BSA) (4,36,85,88,96,97); N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) with TMCS (87,98); and trimethylsilylimidazole (TSIM) (12,16).

The silylation reaction is thought to involve the formation of a transition state such as shown below (99).

\[
HY:+R-Si-X \rightarrow \left[ \begin{array}{c}
\delta^- \\
\delta^+ \\
\delta^-
\end{array} \right]_{\text{Y}} \quad \text{R} \quad \text{R} \quad \text{R} \quad \text{R} \quad \text{R} \quad \text{R} \quad \text{R} \\
H \quad \text{Si} \quad \text{Si} \quad \text{Si} \\
X \quad X \quad X \quad X
\]

The properties most desired of X, the leaving group, are low basicity, the ability to stabilize a negative charge in the transition state and little or no π back-bonding between X and silicon. TSIM and BSA are potent silylating agents because the leaving groups, imidazole and acetamide, can stabilize a partial negative charge through resonance.
The approximate order of silyl donor ability of the agents mentioned above is: TSIM > BSTFA > BSA > TMG > HMDS. The strongest silylating reagent mixture for all purposes is a mixture of BSA: TSIM: TMCS: (1:1:1). Polar solvents tend to increase the rate of the reaction as does increasing the reaction temperature. Moisture decomposes both the reagents and the derivatives. Therefore, the preparation of silyl ethers is usually carried out under anhydrous conditions.

All of the more recent GC references include the addition of an internal standard usually as an initial step in the treatment of the sample. Nalorphine is an excellent choice because of its similarity in structure to morphine and its ability to form the silyl ether derivative (82, 89). In a study of twelve standard solutions, each containing 5 ng/µL of morphine and nalorphine, the relative standard deviation of the absolute morphine peak height was ±23.4% while the morphine/nalorphine peak height ratio only varied ±4.0% (82).

The liquid stationary phases most often used for chromatographic columns in the analysis of morphine are SE-30, a dimethyl silicone non-polar phase, and OV-17, a phenyl methyl silicone mildly polar phase. Moffat suggested the preferred liquid phase for the identification of basic drugs should be SE-30 because it has been used most often and more data is available for it (100). OV-1 and OV-101 are very similar to SE-30 and have been used. These columns
allow the separation of the silyl ethers by boiling point which is the preferred method of separation (99). The support for the liquid phase is silanized. A new column should be conditioned with the derivatizing agent or Silyl-8 column conditioner. Pierce recommends that the chromatograph have glass injection ports since erratic and irreproducible results start occurring after the column has been in use (101). However, he maintains that glass columns are unnecessary. Contrary to this advice the use of glass columns is extensive.

The flame ionization detector (FID) has been most widely used. It is a universal detector. The diacetyl derivative of morphine extracted from bile, blood, liver or urine was injected onto a 3% OV-17(on chromasorb WHP 80/100 mesh) column and nanogram quantities of morphine were determined using an FID detector (92). Wilkinson and Way reported a sensitivity of 25 ng/mL blood using BSTFA as the derivatizing agent, a 3% OV-1 column and FID (87). However, several orders of magnitude in sensitivity can be gained by using an electron-capture detector (ECD). Dahlstrom and Paalzow used a 3H-electron-capture detector in their method which was capable of detecting 100 pg of morphine in 30 mg of brain tissue (94). Nicolau et al. found that 2 pg of the pure derivative was the lowest amount detectable in their urine analysis using the heptafluorobutyryl derivative, a 3% OV-17 column and a 63Ni ECD (95). Gas chromatography with an ECD is as sensitive as RIA but much more specific. HPLC with an electrochemical
detector is as sensitive as GC-ECD and as specific and in addition does not require derivatization (86).

Wilkinson and Way noted that a major problem in the analysis of small quantities of morphine was the tendency of the drug to be adsorbed on the glassware used in the experiment particularly while evaporating the HCl extract to dryness (87). They were unable to eliminate the problem by silanizing, siliconizing or scrupulously cleaning the glassware. They finally used polycarbonate tubes in the concentration step after rejecting polystyrene and polypropylene tubes. Others have chosen to silanize or siliconize all glassware (85,94,102-104). Wallace et al. recommended acid-washing all glassware when morphine in amounts less than 100 ng are being determined (82). Nicolau et al. also recommend acid-washed and distilled water rinsed glassware (95).

The mass spectrometer is a universal detector for GC, since any compound that can pass through a GC will be converted into ions in the MS. At the same time the highly specific nature of mass spectra permits the mass spectrometer to be used as a highly specific GC detector. Its sensitivity is similar to the ECD (105). The procedure recommended by the National Institute on Drug Abuse uses deuterium labeled morphine as the internal standard, a 3% OV-17 column and chemical ionization mass spectrometry using the technique of selected ion monitoring (1). Using this method morphine concentrations as low as 1 ng/mL can be accurately measured. Ammonia is used as the reagent gas.
A similar procedure is described using methane as the reagent
gas and a computer-controlled quadrupole MS to detect concen-
tration as low as 5 ng/mL (106). According to Thenot and Haegele
chemical ionization conditions are usually used for quantitative
work while electron impact ionization is usually used for
identification and structural studies (83).

In a brief survey of the literature for the recovery
of morphine from biological samples analyzed by gas chromo-
tography it is immediately apparent that there is a range
of values, 58 to 95%, depending upon the biological sample
analyzed and the extent of the purification before derivati-
zation. The lowest recovery, 54±12.3% was obtained for
urine samples which were hydrolyzed with β-glucuronidase
and extracted with ether, a rather poor choice of solvent
(96). [14C]-Morphine was added to urine specimens to determine
recovery in this case. When the results of morphine added
to and then extracted from a biological sample and then
derivatized were compared to those when known amounts of
morphine were derivatized directly, the reported recovery
varied from 68% to 95% for urine samples (36,85,92,95).

For liver samples values of 61.2±2.8 and 76.4% were reported
(85,92). A recovery of 70% was reported from rat brain
tissue (94). When nalorphine recovery was reported the
values were 83±2.6% and 98% (36,94).

G. The Study

The intent of this study is to examine the morphine
levels in a tissue that may not be routinely analyzed in
the forensic laboratory, the brain. The brain is of particular interest because of the high concentration of opiate receptors found in various areas of the brain.

Initially aliquots of the standard morphine solution and the nalorphine internal standard solution will be added to water and after adjusting the pH of the solution, a single extraction will be made with the organic solvent. This simplified system will be used to evaluate the most efficient gas chromatographic conditions, to determine the optimum conditions for derivatization and to obtain an estimate of the precision to be expected in the analysis.

After evaluation of the results from the aqueous standards, aliquots of the morphine and internal standard solutions will be added to drug-free brain tissue samples and the entire extraction procedure from biological samples will be developed and evaluated with a recovery study.

Finally, actual brain samples from heroin addicts will be analyzed and the results will be compared to typical data obtained in a toxicology laboratory. The method will be evaluated and the value of brain tissue as an analytical sample in assessing heroin fatalities will be discussed.
CHAPTER II
EXPERIMENTAL

A. Apparatus

A Varian Model 2740 gas chromatograph equipped with a 6-foot x 2mm ID glass column packed with 3% OV-17 on 80/100 mesh Gas-Chrom Q (Applied Science Division, Milton Roy Laboratory Group, State College, PA 16801, Lot SP-2316). The temperature of the injection port was 265°C. The flame ionization detector was maintained at 265°C and the oven was held at 235°C. The carrier gas was nitrogen maintained at a flow rate of about 30 mL/min. The air flow rate was about 275 mL/min and the hydrogen flow rate about 30 mL/min. The GC was connected to an Omniscribe recorder, Model B5217R-15 (Fisher Scientific Co., Itasca, IL 60143). Vespel ferrules (Applied Science) were used to join components of the all glass column system.

The glass columns were washed with a 10% solution of dimethyl dichlorosilane in hexane followed by several methanol washes before being dried and packed. The columns were then conditioned overnight. Silyl-8 (Pierce Chemical Company, Rockford, IL 61105), a column conditioner was injected occasionally. The FID was dismantled and cleaned in a sonic cleaner on a routine basis.

During the initial study using aqueous standards the glass column had a 4mm ID (Supelco, Inc., Bellefonte, PA 16823). As stated above a 2mm ID column (Varian Associates; Sunnyvale, CA 94086) was used in analyzing the actual samples. Lot #SP-2316, 3% OV-17 was used to pack all columns.
B. Materials

1. Chemicals and Reagents

Morphine sulfate, USP (Lot #BRA-056) was obtained from
Merck and Co., Rahway, NJ 07065. Nalorphine hydrochloride was
obtained from Merck, Sharp and Dohme, Rahway, NJ 07065. Stand-
dard solutions of morphine and nalorphine were prepared in
water containing 100μg/mL. Standard solutions of both drugs
were also prepared in methanol.

The Nanograde solvents, chloroform and isopropanol, manu-
factured by Mallinckrodt were supplied by Sargent-Welch
Scientific Co., Skokie, IL 60077. The methanol was Spectrar
grade also purchased from Sargent-Welch Scientific Co. The
pyridine was Silylation grade obtained from Pierce Chemical
Co.

Tri-Sil Z, a solution of trimethylsilylimidazole in dry.
pyridine (1.5 mEq/mL), from Pierce Chemical Company was used
as the silylating agent. Dimethyldichlorosilane was also
purchased from Pierce Chemical Co.

Concentrated HCl, Electronic grade, was purchased from
E. I. DuPont De Nemours and Co., Wilmington, DE 19898. A
40% solution of sodium hydroxide was prepared from Electrolytic
Pellets obtained from Fisher Scientific Company. The above
reagents were also employed to prepare 6M NaOH and 6M HCl.

A 0.5N solution of sulfuric acid was prepared from concentrated
H₂SO₄, Baker Analyzed Reagent, J. T. Baker Chemical Co.,
Phillipsburg, NJ 08865. Potassium bicarbonate and sodium
sulfate were analytical grade reagents.
Whatman phase separating paper, 1PS, was purchased from Fisher Scientific Co.

The pH was tested with pHydron wide range, 0-14, and short range, 8.0-9.5, paper from Sargent-Welch Scientific Co. All aqueous solutions were prepared from doubly-distilled water, the second distillation in an all glass apparatus, Corning AG-1b.

2. Samples

All of the brain samples were supplied by the Wayne County Medical Examiner's Office, Detroit, Michigan. They were obtained from heroin addicts during routine autopsies by various pathologists over a three year period. The brain sections were labeled and frozen. They were transported frozen to Grand Valley State College, Allendale, Michigan where the analyses were carried out.

The brain samples obtained were used for further investigation into the cause of death and therefore satisfy the State of Michigan statutes with respect to use of human body materials. Furthermore, the use of human body materials in research at this university was approved by the Human Research Ethics Committee of the University of Windsor.

C. Method

1. Analytical Studies of Aqueous Standards

The purity of the morphine sulfate used in preparing the standard solutions was checked by ultraviolet spectrophotometry. The absorptivity of a solution containing 10 mg% morphine
dissolved in 0.1N H₂SO₄ was determined at the wavelength of maximum absorption using a Cary 14. A holmium oxide glass was used to check the wavelength accuracy of the Cary 14. The absorbance accuracy of the instrument was checked by dissolving 0.0500g of primary standard grade potassium dichromate in 0.005M H₂SO₄. The absorptivity of this solution at 350nm should be 10.70 Lg⁻¹cm⁻¹ (107). A 1% solution of morphine in 0.1N H₂SO₄ is reported to have an absorptivity of 55 at 287 nm (108).

Optimum extraction and chromatographic conditions were determined by adding aliquots (100μL to 1000μL) of the standard morphine solution and/or aliquots (100μL to 500μL) of the standard naltorphine solution to 5mL of distilled water, adjusting the pH and extracting the morphine and/or naltorphine with 5 mL of the organic solvent. The aqueous layer was aspirated and discarded. The organic layer was then filtered through silanized glass wool containing anhydrous sodium sulfate or through phase separating paper, Whatman 1PS, containing anhydrous sodium sulfate. The solvent was evaporated in a thermal block at about 65°C under a gentle stream of dry nitrogen. The bis(trimethylsilyl) derivatives were formed, the conditions of the derivatization depending upon the derivatizing agent used. In all cases, a 1μL sample was injected into the GC.

2. Recovery Study

Aliquots (10μL, 20μL and 50μL) of the 100μg/mL morphine standard solution in methanol were added in triplicate to screw-capped septum vials followed by 250μL of the internal standard solution. The methanol was evaporated and the sample
derivatized with 50 μL of Tri-Sil Z. A 1-μL aliquot of this solution was injected into the GC and a standard curve drawn by using linear regression analysis. Brain samples, to which identical amounts of morphine and nalorphine were added and then extracted and derivatized by the procedure described below (CHAPTER II, Section C.3. Biological Samples), were chromatographed and read from the standard curve to determine the extraction efficiency.

3. Analysis of Biological Samples

Each frozen brain section was weighed, an equivalent volume of distilled water added and the sample homogenized in a Waring blender equipped with stainless steel mini sample containers. If sufficient sample was available a 10 to 15g aliquot of the homogenate was taken. Otherwise the entire sample was used. A 250-μL aliquot of the standard nalorphine hydrochloride solution containing 100 μg nalorphine per mL was added to each sample and vortexed. Sufficient concentrated HCl was added to each sample to obtain a concentration of 11% HCl in the final mixture. The samples were then hydrolyzed for 30 min in a boiling water bath. After cooling, the mixtures were neutralized by the slow addition of a 40% NaOH solution. The pH was adjusted to 8.7 ± 0.2 (8.0–9.5 pHydron paper) and a spatula of approximately 0.5 g KHCO₃ was added and the mixture vortexed. To this mixture 20 mL of chloroform-isopropanol (4:1) were added and the samples placed on a rotator for 15 min (19 rpm). The aqueous layer was aspirated and discarded. The organic layer was filtered through solvent
washed Whatman 1FS paper containing approximately 0.5g of powdered anhydrous sodium sulfate. To the filtrate 5mL of 0.5N H₂SO₄ were added and the samples rotated for 15 min and then centrifuged for 15 min. The aqueous layer was removed by pipet and the pH of this layer adjusted to 8.7±0.2 by saturating the solution with KHCO₃ and adding 0.10mL 6N NaOH. To this basic aqueous layer 5mL of chloroform-isopropanol (4:1) solution were added followed by rotation for 15 min and then centrifugation for 10 min. The aqueous layer was aspirated and discarded while the organic layer was filtered through 1FS paper containing Na₂SO₄ into a screw-capped septum vial equipped with a Teflon-lined stopper. The residue was dissolved in 50µL of Tri-Sil-Z and placed in the thermal block at 90-95°C for 30 min. Within 15 min 1µL was injected into the GC under the standard conditions.

With each set of samples a set of standards was prepared by adding known quantities of morphine and the internal standard to brain tissue samples that were drug-free. These standards were processed identically to the samples being analyzed. The ratio of the peak height of the morphine/nalorphine was then plotted vs the concentration of morphine added. The concentration of morphine in the samples was then read from this curve.

Formation of only the bis(trimethylsilyl) morphine derivative was confirmed by GC-MS. The gas chromatogram and the mass spectrum of the derivative are shown in the Appendix.
CHAPTER III
RESULTS AND DISCUSSION

A. Analytical Studies of Aqueous Standards

The absorptivity calculated from the absorbance measured for a 10 mg % solution of morphine sulfate dissolved in 0.1N H₂SO₄ was in agreement with that found in the literature indicating that the compound was of acceptable purity as received from the supplier (64, 108).

In the extraction procedure after the morphine and/or nalorphine had been added to the 5mL of distilled water the pH was adjusted to optimize the extraction of the drugs into the organic layer. After trying K₂CO₃ (pH>10) and a mixture of K₂CO₃-KHCO₃ (3:8) giving a pH of 9.6 it was determined that KHCO₃ alone was most suitable. Since the isoelectric point of morphine occurs at a pH of 8.9, morphine has zero net charge at this pH favoring extraction into the organic layer.

Potassium carbonate has been used by Fujimoto and Way to separate morphine and its glucuronide from urine (109). It has also been used by Kohn-Abrest to saturate distillates of blood (110). If ethanol was present in these distillates, it formed a separate layer. The volume of this layer was used to quantitate the ethanol. This suggested to Bastos et al. that if urine is saturated with potassium carbonate the addition of ethanol could be used to concentrate the drugs present in urine (66). Horning et al. have substituted ammonium carbonate and various other solvents in isolating drugs from biological fluids (111). These authors believe the major
advantage of salt-solvent extraction procedures is that emulsions do not form. In this study KHCO₃ was used to adjust the pH, saturate the aqueous layer with salt and decrease the solubility of the isopropanol in the aqueous layer.

Wallace et al. had reported that chloroform provided a 25% better recovery of morphine from plasma than methylene chloride (82). The recovery was even better when isopropanol or isobutanol was added to the chloroform. During this study three different ratios of chloroform to isopropanol were used: 9 to 1, 4 to 1 and 3 to 1. The solvent ratio finally chosen was 4 to 1 because it was purported to extract approximately 90% of the free morphine in a single extraction when the sample to solvent ratio was 1:4(80). Incorporating higher molecular weight alcohols such as isobutanol or n-pentanol would increase the efficiency of the extraction slightly but also increase the temperature for evaporation of the solvent.

After extracting the morphine and/or nalorphine into the organic layer the bulk of the aqueous layer was aspirated off and discarded. To remove the last traces of moisture from the organic layer two procedures were evaluated. The organic layer was filtered through silanized glass wool containing anhydrous Na₂SO₄ or it was filtered through solvent washed phase separating paper, Whatman 1PS, containing anhydrous Na₂SO₄. Although trace amounts of silicone and the catalyst used in the production of the paper were extracted in the latter process it was judged to be the more efficient procedure.
In the former process the amount of glass wool used in the filtration was a critical factor. If the quantity was too small salt and/or water was carried into the derivatizing vial. If the quantity was too large significant sample was trapped in the glass wool.

Several runs were made using cholestane as the internal standard instead of nalorphine. There was no advantage to be gained in using cholestane since it took longer to elute from the GC column and did not serve as a check in the derivatization step since it does not form a derivative.

After evaporation of the organic solvent the bis(tri-methylsilyl) derivative was formed using either a 40% solution of BSA in pyridine or Tri-Sil Z as the derivatizing agent. If BSA was used the sample was heated for 15 min at 60°C or allowed to stand at room temperature for varying periods of up to one hour. When Tri-Sil Z was used the sample was heated for 30 min or 1 h at 90-95°C. The desired derivative was formed with both derivatizing agents. Tri-Sil Z was the agent of choice because it was recommended as capable of silylating in the presence of moderate amount of water (101). Various volumes (25μL-200μL) of the derivatizing agent were used. It is essential that sufficient excess be present to react with all of the morphine and/or nalorphine after any hydrolysis due to the presence of moisture has occurred. Too much Tri-Sil Z is to be avoided as it acts as a diluent. At the morphine concentrations found in the brain samples it was determined by experiment that 50μL of Tri-Sil Z was sufficient
for complete derivatization.

In chromatographing the samples the major variable investigated was column temperature. The goal was to elute the morphine and nalorphine as soon as possible after the solvent front with good resolution. Isothermal temperatures ranging from 220 to 280°C were used as well as temperature programming from 200°C to 280°C and 220°C to 280°C at a rate of 6°C/min. Temperature programming was effective but was very time consuming as the temperature had to be stabilized at the base temperature between each run or erratic results were obtained. The most effective separation was obtained at a column temperature of 235°C.

Table II is a summary of the within-day variation obtained in the analysis of morphine extracted from water solution. Table III summarizes the between-day variation. The data summarized in Table III is graphed in Fig 7. Figure 8B illustrates a typical chromatogram obtained after the extraction of morphine from aqueous solution.

B. Recovery Study

In the recovery study, 10μL, 20μL and 50μL of the internal standard morphine solution containing 100μg of morphine/mL in methanol and 25μg of the nalorphine internal standard solution were prepared in triplicate and evaporated to dryness before derivatizing with 50μL of Tri-Sil Z. These solutions contained 1, 2 and 5μg of morphine, respectively. Figure 8A is an example of the chromatograms obtained in the recovery study. Since only
TABLE II
ANALYSIS OF MORPHINE EXTRACTED FROM WATER SOLUTION
WITHIN-DAY VARIATION

<table>
<thead>
<tr>
<th>Morphine added (µg/mL)</th>
<th>#Samples</th>
<th>Peak height ratio (M/N) ( ^a ) mean (±S.D.) ( ^b )</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6</td>
<td>8</td>
<td>0.133±0.011</td>
<td>8.3%</td>
</tr>
<tr>
<td>3.2</td>
<td>8</td>
<td>0.267±0.003</td>
<td>1.2%</td>
</tr>
<tr>
<td>8.0</td>
<td>8</td>
<td>0.704±0.015</td>
<td>2.1%</td>
</tr>
</tbody>
</table>

\( ^a \) M - morphine peak height
\( ^N \) N - nalorphine peak height

\( ^b \) S.D. - standard deviation
### TABLE III
ANALYSIS OF MORPHINE EXTRACTED FROM WATER SOLUTION:
BETWEEN-DAY VARIATION

<table>
<thead>
<tr>
<th>Morphine added (µg/mL)</th>
<th>#Samples</th>
<th>Peak height ratio (M/N) (^a) mean (±S.D.) (^b)</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6</td>
<td>10</td>
<td>0.124±0.024</td>
<td>19%</td>
</tr>
<tr>
<td>3.2</td>
<td>9</td>
<td>0.299±0.026</td>
<td>8.7%</td>
</tr>
<tr>
<td>4.8</td>
<td>2</td>
<td>0.454±0.006</td>
<td>1.3%</td>
</tr>
<tr>
<td>8.0</td>
<td>9</td>
<td>0.787±0.073</td>
<td>9.3%</td>
</tr>
</tbody>
</table>

\(^a\)M - morphine peak height  
\(^b\)N - nalorphine peak height  
\(^b\)S.D. - standard deviation
FIGURE 7
STANDARD CURVE: MORPHINE EXTRACTED FROM WATER SOLUTION

Legend

Aliquots of a standard morphine and nalorphine solution were added to water and extracted and derivatized as described in CHAPTER II, Section C.1. Analytical Studies of Aqueous Standards. Table III summarizes the data obtained after gas chromatography of the derivatives.

Linear regression analysis of the data in Table III resulted in the figure as illustrated.

Slope: 0.103
Intercept: -0.038
Correlation coefficient 0.99

Mean ± S.D.
FIGURE 7

STANDARD CURVE: MORPHINE EXTRACTION FROM WATER SOLUTION

MORPHINE/NALORPHINE PEAK HEIGHT RATIO

MORPHINE ADDED (µg)
FIGURE 8
REPRESENTATIVE CHROMATOGRAMS

Legend

Chromatograms were obtained by injecting 1μL of the samples described below.

A: 50μL of a 100μg/mL morphine solution in methanol and 250μL of a 100μg/mL naltrexone solution in methanol were evaporated to dryness and derivatized with 50μL of Tri-Sil Z.

B: 50μL of a 100μg/mL morphine solution and 250μL of a 100μg/mL naltrexone solution were added to 5 mL H₂O, the pH adjusted and the drugs extracted with 5 mL of CHCl₃/isopropanol (4:1), evaporated and derivatized.

C: 50μL of a 100μg/mL morphine solution and 250μL of a 100μg/mL naltrexone solution were added to a 10g drug-free brain homogenate and treated as described under Biological Samples (CHAPTER II, Section C).

D: 250μL of a 100μg/mL naltrexone solution were added to 13.568g of sample #12-82 medulla homogenate and treated as described under Biological Samples (CHAPTER II, Section C).

The chromatograms were run on a 3% OV-17 column. The column temperature was 235°C. The injection port and detector were maintained at 265°C. The nitrogen carrier gas flow rate was about 30mL/min, the air flow about 275mL/min and the hydrogen flow about 30mL/min. Chart speed was 5mm/min.

a - Morphine
b - Nalorphine
FIGURE 8
REPRESENTATIVE CHROMATOGRAMS
1 μL of the derivatized sample was injected onto the column the actual concentration of morphine represented by the morphine peak (a) in Chromatogram A is 0.10 μg. Table IV summarizes the results obtained in the recovery study. The second column of Table IV lists the mean and standard deviation for the morphine/nalorphine peak height ratios for each concentration of the methanol standards. Linear regression analysis of this data resulted in Fig. 9, a calibration curve for the data. These same concentrations of morphine and the internal standard were added to drug-free samples of homogenized brain tissue and analyzed according to the specified procedure, over a period of several days (subject to between-run variation as opposed to within-run variation for the standards).

The mean and standard deviation of the morphine/nalorphine peak height ratios are shown in the third column of Table IV. The concentrations corresponding to these peak height ratios were then read from the calibration curve. The amount of morphine remaining in the sample after the extraction process was determined in this manner. The mean recovery was 78%. No correction has been made for solvent loss. The morphine peak in Chromatogram C of Fig. 8 thus represents an actual concentration of 0.078 μg of morphine (5 μg/50 μL x 1 μL x 0.78).

C. Analysis of Biological Samples

The procedure used in the analysis of the brain sample was designed to incorporate the best features found in the literature for the extraction process with simplicity and
### TABLE IV

RECOVERY OF MORPHINE

<table>
<thead>
<tr>
<th>Morphine added</th>
<th>Methanol standards</th>
<th>Brain samples</th>
<th>Morphine recovered</th>
<th>Percent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μg</td>
<td>0.041</td>
<td>0.029</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.044</td>
<td>0.032</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.044</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean(±S.D.)</td>
<td>0.043±0.002</td>
<td>0.030±0.002</td>
<td>0.70</td>
<td>70%</td>
</tr>
<tr>
<td>2 μg</td>
<td>0.0943</td>
<td>0.0967</td>
<td>2.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.101</td>
<td>0.0973</td>
<td>1.48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0876</td>
<td>0.0600</td>
<td>1.32</td>
<td></td>
</tr>
<tr>
<td>Mean(±S.D.)</td>
<td>0.0943±0.007</td>
<td>0.0747±0.019</td>
<td>1.63</td>
<td>80%</td>
</tr>
<tr>
<td>5 μg</td>
<td>0.226</td>
<td>0.165</td>
<td>3.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.237</td>
<td>0.201</td>
<td>4.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.237</td>
<td>0.214</td>
<td>4.74</td>
<td></td>
</tr>
<tr>
<td>Mean(±S.D.)</td>
<td>0.233±0.006</td>
<td>0.193±0.025</td>
<td>4.19</td>
<td>84%</td>
</tr>
</tbody>
</table>
FIGURE 9
CALIBRATION CURVE USING METHANOL STANDARDS

Legend
Linear regression analysis of the data in TABLE IV for the recovery of morphine resulted in this calibration curve.

Slope 0.0473
Intercept -0.0026
Correlation Coefficient 0.998

Mean ± S.D.
FIGURE 9
CALIBRATION CURVE USING METHANOL STANDARDS
efficiency. No attempt was made to wash the organic layer with buffer solution to remove any water soluble contaminants that might have been carried over into the organic layer. Nor was the acidic aqueous layer washed with an organic solvent such as hexane to remove possible organic contaminants. Both of these procedures could lead to "cleaner" chromatograms but also to a loss of morphine and increased analysis time.

Since the major metabolite of heroin, morphine-3-glucuronide, is water soluble biological samples must be hydrolyzed before the total morphine concentration can be determined. Although either enzymatic or acid hydrolysis is possible, enzymatic hydrolysis is a much more time-consuming process. Combie et al. have reduced the time to 3 h at 65°C using 8-glucuronidase from Patella vulgata to completely hydrolyze the morphine glucuronide in urine samples (112). However, Ebbighausen et al. have found that the maximum yield of morphine and 6-monoacetylmorphine in blood samples was obtained in 30 min at 100°C using acid hydrolysis when the HCl concentration was 4N(113). If 4N (\(\sim\)12%) HCl was used the yield decreased with time after 30 min. If 3N HCl was used the yield increased with time. In 120 min the yield obtained using 3N HCl was not quite equal to that obtained in 30 min using 4N HCl.

After acid hydrolysis the pH of the solution was originally adjusted to pH 8.7 \(\pm\) 0.2 with concentrated NH\(_3\) and KHCO\(_3\) before the initial extraction with organic solvent. Often during this period the chromatograms obtained after derivat-
tization did not show peaks where the derivatized drugs should have eluted. Believing moisture to be the cause of this problem attempts were made to eliminate all moisture by substituting anhydrous magnesium sulfate for anhydrous sodium sulfate when filtering the organic layer through 1 PS paper, adding benzene to the sample vial before evaporating the organic solvent and drying the sample in a vacuum oven for 2h at 65°C before derivatization. Eventually the problem was eliminated by substituting 40% NaOH for the NH₃ in neutralizing the acid hydrolysate.

In the derivatization step the excess solvent could have been evaporated and the sample dissolved in a smaller volume of another solvent, such as ethyl acetate or methanol. This would increase the morphine concentration in the injected sample but introduces another step in the procedure. Pyridine has a tendency to tail badly in chromatograms and can interfere with the analysis of volatile substances (99). As can be seen in Fig. 8, there is some tailing of the solvent in this analysis but it does not interfere with quantification.

Whenever a set of samples was run a set of controls was also run. The controls consisted of three or four drug-free brain homogenates to which morphine was added. A blank containing only the internal standard was always included in these controls. The morphine/nalorphine peak height ratios were determined and plotted vs the concentration of morphine originally added. The concentration of morphine in the samples
was then determined directly from the graph and divided by the weight of the tissue in the original homogenate to find the morphine concentration per gram of brain tissue. After all of the samples were run linear regression analysis was used to obtain a composite curve of brain control standards from 14 runs (see Fig. 10).

The results of the analysis of the brain samples obtained from the Wayne County Medical Examiner's Office are presented in Table V. All of the data presented at the bottom of the table (below the double horizontal lines) was supplied from the Toxicology Laboratory of the Medical Examiner's Office through the courtesy of Dr. Joseph Monforte. This data was received after the samples were analyzed. The samples were numbered in the order in which they were analyzed and the year during which they were obtained and are not the actual case numbers.

In the interpretation of these results it is necessary to bear in mind that the samples were obtained over a number of years by various pathologists. As a result there is no doubt a wider variation in the actual location from which the brain specimens have been excised than might seem to be indicated by the label given the specimen. For example, the \( \mu \)-receptors in the thalamus are concentrated in the dorso-medial nucleus of the thalamus. This nucleus may or may not have been included in its entirety in the sample received for analysis.
FIGURE 10

COMPOSITE CURVE: BRAIN CONTROL STANDARDS

Legend

After all of the brain samples had been analyzed, the data from the calibration curves from each analysis were pooled. The results are tabulated below. Linear Regression analysis of this data resulted in the composite curve in this figure.

<table>
<thead>
<tr>
<th>Morphine added</th>
<th>Morphine/nalorphine peak height ratio Mean ± S. D.</th>
<th>Number of runs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1µg</td>
<td>0.030±0.002</td>
<td>4</td>
</tr>
<tr>
<td>2µg</td>
<td>0.084±0.016</td>
<td>14</td>
</tr>
<tr>
<td>5µg</td>
<td>0.218±0.025</td>
<td>14</td>
</tr>
</tbody>
</table>

Slope 0.452
Intercept -0.008
Correlation coefficient 0.965

Mean ± S.D.
FIGURE 10

COMPOSITE CURVE: BRAIN CONTROL STANDARDS

MORPHINE/NALORPHINE PEAK HEIGHT RATIO

MORPHINE ADDED (μg)
<table>
<thead>
<tr>
<th>Brain portion</th>
<th>1-79</th>
<th>2-79</th>
<th>3-79</th>
<th>4-00</th>
<th>5-01</th>
<th>6-01</th>
<th>7-01</th>
<th>8-01</th>
<th>9-01</th>
<th>10-02</th>
<th>11-02</th>
<th>12-02</th>
<th>13-02</th>
<th>14-02</th>
<th>15-02</th>
<th>16-02</th>
<th>17-02</th>
<th>18-02</th>
<th>19-02</th>
<th>20-02</th>
<th>21-02</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain stem</td>
<td>0.75</td>
<td>0.34</td>
<td>0.14</td>
<td>0.013</td>
<td>0.17</td>
<td>0.16</td>
<td>0.19</td>
<td>0.16</td>
<td>0.14</td>
<td>0.52</td>
<td>0.11</td>
<td>0.14</td>
<td>0.28</td>
<td>0.14</td>
<td>0.28</td>
<td>0.34</td>
<td>0.14</td>
<td>0.28</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medulla</td>
<td>0.04</td>
<td>0.20</td>
<td>0.20</td>
<td>trace</td>
<td>0.20</td>
<td>trace</td>
<td>0.20</td>
<td>0.20</td>
<td>trace</td>
<td>0.20</td>
<td>0.13</td>
<td>trace</td>
<td>0.16</td>
<td>0.36</td>
<td>0.15</td>
<td>0.35</td>
<td>0.13</td>
<td>0.28</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Midbrain</td>
<td>0.55</td>
<td>0.52</td>
<td>0.15</td>
<td>0.14</td>
<td>0.25</td>
<td>trace</td>
<td>0.37</td>
<td>0.23</td>
<td>0.20</td>
<td>trace</td>
<td>0.25</td>
<td>0.47</td>
<td>0.20</td>
<td>0.28</td>
<td>0.43</td>
<td>0.14</td>
<td>0.25</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.53</td>
<td>0.53</td>
<td>0.27</td>
<td>0.18</td>
<td>0.20</td>
<td>0.19</td>
<td>0.31</td>
<td>0.22</td>
<td>0.32</td>
<td>0.23</td>
<td>0.099</td>
<td>0.21</td>
<td>0.25</td>
<td>0.24</td>
<td>0.33</td>
<td>0.07</td>
<td>0.21</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>0.55</td>
<td>0.53</td>
<td>0.39</td>
<td>0.27</td>
<td>0.16</td>
<td>0.15</td>
<td>0.16</td>
<td>0.29</td>
<td>0.19</td>
<td>trace</td>
<td>0.31</td>
<td>0.45</td>
<td>0.21</td>
<td>0.22</td>
<td>0.36</td>
<td>0.17</td>
<td>0.20</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal ganglion</td>
<td>0.62</td>
<td>0.64</td>
<td>0.14</td>
<td>0.41</td>
<td>trace</td>
<td>0.09</td>
<td>0.18</td>
<td>0.04</td>
<td>0.24</td>
<td>0.27</td>
<td>0.13</td>
<td>0.40</td>
<td>0.04</td>
<td>0.28</td>
<td>0.31</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>0.67</td>
<td>0.64</td>
<td>0.14</td>
<td>0.28</td>
<td>0.24</td>
<td>0.12</td>
<td>0.15</td>
<td>0.18</td>
<td>0.28</td>
<td>0.18</td>
<td>0.05</td>
<td>0.29</td>
<td>0.19</td>
<td>0.29</td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.67</td>
<td>0.64</td>
<td>0.14</td>
<td>0.28</td>
<td>0.24</td>
<td>0.12</td>
<td>0.15</td>
<td>0.18</td>
<td>0.28</td>
<td>0.18</td>
<td>0.05</td>
<td>0.29</td>
<td>0.19</td>
<td>0.29</td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Blood Morphine: 0.76, 2.40
Urine Morphine: 0.008, 0.28, 0.24
Morphine-post: Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile

Blood ethanol: 0.00%, 0.00%
Urine ethanol: 0.00%, 0.00%
Blood quinine: 2.8, 5.3, 4.4
Urine quinine: + + +
Quinine-positive: Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile, Bile

*Morphine Concentration in ug/ sample
With one exception (case 2-79) the concentration of morphine found in the brain was approximately equal to or higher than that found in the blood. The blood was analyzed by a spectrofluorometric method (114). When the highest concentration found in the brain was compared to that in the blood, where numerical values were available, the mean ratio was 2 to 1. This data is displayed graphically in Fig. 11. The results of the linear regression analysis of the data in Table V as well as that in Fig. 11 are summarized in the legend to Fig. 11.

In over half of the samples analyzed the highest concentration of morphine was found in the brain stem or the thalamus. These are two areas in the brain where many of the adverse effects of heroin or morphine originate. One of the unpleasant side effects of morphine or heroin is the nausea and vomiting caused by stimulation of the chemoreceptor trigger zone for emesis located in the area postrema of the medulla which comprises the base of the brain stem. In cases of heroin overdose the cause of death is nearly always due to respiratory arrest (115). The nuclei which are reflex centers for the regulation of respiratory rate and also heart rate are located in the brain stem. Morphine decreases the response of the respiratory centers to increases in carbon dioxide tension, depresses respiratory rhythm and the response to electrical stimulation. Opiates are used to depress the cough reflex which is also located in the medulla. The characteristic pinpoint pupils of an addict are primarily
FIGURE 11
COMPARISON OF MORPHINE CONCENTRATION IN BRAIN AND BLOOD

Legend

In this figure the highest concentration of morphine found in the brain is compared to that in the blood of each addict. The straight line resulted from linear regression analysis of this data. The brain samples were analyzed by GC and the blood samples were analyzed, by the Wayne County Medical Examiner's Office, using spectrofluorometry.

The numbers in the figure correspond to the sample numbers as given in Table V on page 77.

In the table below linear regression analysis was used to evaluate the relationship of the concentration of morphine in the blood (X values) to that in each of the analyzed brain sections (Y values).

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Slope</th>
<th>Y-Intercept</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highest brain concentration</td>
<td>13</td>
<td>0.46</td>
<td>0.24</td>
<td>0.54</td>
</tr>
<tr>
<td>Brain stem</td>
<td>13</td>
<td>0.78</td>
<td>0.092</td>
<td>0.61</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>11</td>
<td>0.48</td>
<td>0.16</td>
<td>0.45</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>12</td>
<td>-0.14</td>
<td>0.26</td>
<td>-0.20</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>13</td>
<td>0.05</td>
<td>0.24</td>
<td>0.49</td>
</tr>
<tr>
<td>Thalamus</td>
<td>10</td>
<td>1.2</td>
<td>0.024</td>
<td>0.88</td>
</tr>
</tbody>
</table>

aIn the statistical analysis samples 1-79 and 2-79 were rejected as outliers on the basis of Dixon's gap test (p>0.01) and Chauvenet's criterion. (see Quality Control Seminar Notes compiled by Gordon G. Cryumble, Warner-Chilcott Diagnostics, Scarborough, Ontario, p. 28).
due to an effect on the Edinger-Westphal nucleus of the mid-brain. The decrease in urinary output associated with addiction is mediated by the supraoptic nucleus of the hypothalamus. It is thought that opiate agonists exert their analgesic effect at several sites in the brain including the amygdala and hippocampus of the limbic system, the diencephalon and the midbrain.

It is of interest to compare the brain concentration of morphine found in this study to the amounts found to saturate the opiate receptor sites in opiate receptor binding assays. In the adult human brain, the water content averages about 78% and protein comprises about 40% of the remaining solids (116). If 0.25 μg/g of brain tissue is taken as the representative concentration of morphine in the brain tissue of heroin addicts, it would be equivalent to about 10 pmol morphine/mg protein. The saturation amount of opiate-reported by Simon and Hiller is about 0.25 pmol/mg protein in whole brain homogenates (42). This would indicate that the concentration of morphine found in the brain tissue of addicts exceeds that required to bind the opiate receptors by a factor of about 40. This estimate does not take into consideration the fact that the number of opiate receptors is highly concentrated in very specific areas of the brain nor that there are different types of opiate receptors.

D. Case Histories

A total of 21 cases were evaluated. Of these, three cases were found to be essentially morphine-free. Case 15-82 was
found drowned in a bathtub with a syringe under the body and needle tracks on the body. However, no morphine was found in brain tissue or blood. Case 17-82 died of a shotgun wound. There appeared to be a trace of morphine in the occipital lobe of the cerebrum but the level was at the limit of detection and all other brain segments and blood were negative. Case 20-82 died of natural causes and no morphine was detected. The remaining cases for which additional information is available are discussed generally in order of increasing blood morphine concentration.

Case 10-82 died as a result of a gunshot wound to the head. Morphine was not found in the blood but was found in the urine, bile and in several sections of the brain at levels beneath the lethal concentration.

Case 3-79 was found dead in bed 14 h after last being seen. There was a history of drug abuse and drug paraphernalia were found with the body. All blood and urine tests were negative but lethal concentrations of morphine were found in the brain.

Morphine was not identified in the blood of Case 7-81 but was found in several sections of the brain. The chromatograms also showed two large unidentified peaks that were not present in any of the other cases. Two drugs were found in the blood during toxicological evaluation, pentazocine, a benzomorphan derivative with both agonist and weak antagonist activity, and tripelennamine, an antihistamine. The unidentified peaks may have resulted from the presence of
these drugs. The deceased had a history of heroin abuse so it is possible that morphine was present in the brain from a prior injection. Since no other tests were performed on brain tissue containing these drugs no further comments are appropriate.

Although Case 14-82 had a blood morphine concentration well below that considered to be lethal several sections of the brain contained concentrations in excess of 0.20μg/g sample. Alcohol was also detected in the blood. The victim was pronounced dead within 30 min of arrival at the hospital with no additional information available.

Case 4-80 was found dead in bed fully clothed with a sock around one arm and a syringe and needle nearby. Although only the hypothalamus contained sufficient morphine (0.27μg/g sample) to be considered at a lethal level, the presence of alcohol could well have contributed to what appeared to be a relatively rapid death.

Cases 11-82, 13-82, and 18-82 all had blood morphine levels greater than 0.1μg but less than 0.2μg/g sample. Alcohol was present in the blood of Cases 13-82 and 18-32 but not in Case 11-82. Case 11-82 had the highest brain morphine concentration of the three cases but all three had brain levels greater than 0.20μg/g sample in one or more brain section. Case 13-82 died in the hospital emergency room less than one hour after admission. Case 18-82 was dead within 10 min of arrival at the hospital. Case 11-82 died in the bathroom under circumstances that might indicate death.
occurred soon after injection.

Case 8-81 was discovered unconscious by a friend when the friend "came around" after they both had injected heroin. The blood level, 0.12µg/g sample, was below the level normally considered fatal but several brain sections exceeded the lethal level. It's likely that several hours elapsed between injection and discovery, allowing sufficient time for the friend to recover. The victim was on leave from military service and may have lost a prior tolerance to the drug which spared his friend. He died within 15 min of hospital admission.

Cases 12-82, 19-82 and 21-82 had blood morphine levels of 0.28 or 0.29µg/g sample and brain morphine levels which also exceeded the lethal levels in one or more of the sections analyzed. Death appeared to occur soon after injection in all cases. Case 12-82 was found dead on the floor with a syringe in the right hand. Case 21-82 collapsed after injection of the drug before witnesses and died shortly after arrival at the hospital. Case 19-82 died with a tourniquet on his arm and a needle at hand in an automobile parked in the area only a few hours earlier.

Cases 5-81 and 6-81 were found unconscious in the same house and died shortly after arrival at the hospital. Both had lethal levels of morphine in their brain and blood as well as high alcohol concentration in the blood and urine.

Cases 1-79 and 2-79 were found dead in the same room. These victims had the highest brain concentrations of morphine
encountered in this study. The blood morphine concentration in one of the individuals was approximately six times that found in any other case. Alcohol was also present in both the blood and urine of the victims. Death is known to have occurred within three hours of injection of the drug but based on evidence at the scene probably occurred within the hour.
CHAPTER IV
SUMMARY AND CONCLUSIONS

A. Analytical Studies of Aqueous Samples

A relatively simple and efficient procedure for the extraction and derivatization of morphine has been described. This procedure could easily be adapted to the routine analysis of morphine in a toxicology laboratory.

Solid KHCO$_3$ plus several drops of dilute NaOH is an effective means of saturating the solution with salt and adjusting the pH of the solution without the preparation of large amounts of buffer solutions or solid mixtures. The solubility of KHCO$_3$ is greater than that of NaHCO$_3$ but either salt could be used.

A larger volume of the organic solvent would increase the amount of morphine extracted from the aqueous solution. Predmore et al. recommend a solvent ratio of 4 to 1 for a 90% recovery with a single extraction (80). However in this procedure a compromise was made based on the volume limit of the septum vials, evaporation time and the desired minimum detectable level.

Rather than increasing the volume of the solvents which are expensive and in the case of chloroform hazardous, it would be more efficient to evaporate the sample to dryness after the derivatization and then redissolve the residue in a smaller volume of another solvent such as ethyl acetate. The derivative could be easily concentrated fivefold in
this step and at the same time the trailing solvent effect caused by pyridine in the chromatogram would be eliminated.

The precision in replicate measurement could be increased by substituting electronic integration of the peaks for the manual measurement of peak-heights used in this study.

B. Recovery Study

The mean recovery obtained by the procedure described was 78%. As mentioned above this recovery could be improved by increasing the organic solvent to sample ratio. It could also be improved by a second extraction or by substituting a higher molecular weight alcohol for the isopropanol (80). Such techniques are recommended if it is necessary to detect lower levels of morphine in the samples. However, since the water content of the brain averages about 78% it might be advantageous to decrease the water added to the brain sample by as much as 50% when homogenizing the samples and evaluate the effect of concentrating the morphine in this manner on the entire procedure.

C. Analysis of Biological Samples

In this study heroin and its metabolites were the only drugs of interest so there was no need to be concerned about the decomposition of other drugs that might be present when the glucuronides were hydrolyzed at 100°C with HCl. If other drugs are of interest it might be worthwhile to hydrolyze with β-glucuronidase from Patella vulgata which completely hydrolyzed morphine glucuronide in urine at 65°C in 3 h (112).
If additional brain samples are analyzed in the future, arrangements should be made with one pathologist who is willing and able to spend the considerable amount of extra time during autopsy to obtain exactly the same portions of each brain for analysis. This would probably mean that the brain sections would be smaller and perhaps require greater sensitivity in the analysis.

Using the procedure as described, including analysis by GC with a flame ionization detector the minimum detectable quantity of morphine is about 1 μg (0.2 μg morphine/g of brain tissue for a 5 g sample). A one-microliter injection of the morphine derivative onto the GC therefore represents about 0.016 μg of morphine. To increase the sensitivity of the method the easiest modification in the procedure would be to evaporate the derivatizing solvent to dryness and dissolve the derivative in a smaller volume of solvent.

The question arises as to whether the method described is the most effective method for the analysis of morphine in biological samples. Although the method was sufficiently sensitive to analyze the brain sections in this study it would be desirable to be able to analyze very specific sections in the brain which might weigh 1 g or less. Since an ECD is several orders of magnitude more sensitive than the FID it is possible to extract the morphine as described in this procedure, derivatize with a halogenated derivatizing agent and use GC but substitute an ECD for the FID.

When the brain samples were homogenized in the first
step of this procedure if there was any homogenate remaining after the sample was weighed, it was frozen. It is anticipated that as soon as funds are available an electrochemical detector will be purchased to be used with a Varian Model 5000 liquid chromatograph to analyze these samples using a method similar to that described by Sutheimer et al. or Vandenberghhe et al. (86, 117). In the first case 3 mL of blood were analyzed by reverse phase HPLC with a detection limit of 5ng/mL while in the latter case 100uL of serum were analyzed by reverse phase paired-ion HPLC with a detection limit of 1ng/mL. Both procedures used electrochemical detection. The data obtained by HPLC could then be compared to the data reported here for the GC analysis.

D. Case Histories

In the samples analyzed the concentration of morphine in the various areas of the brain was generally equal to or greater than that found in the blood and in some samples was more than twice the concentration in the blood.

It is of interest then to test each of the correlation coefficients (see Fig. 11, Legend, p. 79) determined for the data from Table V against the null hypothesis that the population correlation is zero. This can be done by use of the formula given below. Alternately, there are tables available that give

\[ t_{n-2} = \frac{r}{(1-r^2)^{1/2}}(n-2)^{1/2} \]

- Critical values of the t distribution can be found in statistical tables for various levels of significance and n-2 degrees of freedom
- Correlation coefficient
the sample correlation required to achieve statistical significance at various levels (118).

For 11 degrees of freedom the value of \( r \) required for two-tailed tests at the 5 percent level of significance is 0.553. The correlation coefficients for brain sections with blood morphine concentration in this study having 11 degrees of freedom are: the hypothalamus with \( r \) equal to 0.49, the highest brain concentration with \( r \) equal to 0.54 and the brain stem with \( r \) equal to 0.61. The correlation coefficient between the concentration of morphine in the blood and that in the brain stem is significant at the 5 percent level.

At 8 degrees of freedom the critical value for \( r \) at the 1 percent level of significance is 0.765. The value of the correlation coefficient obtained in this study for the morphine level in blood and that in the thalamus was 0.88.

This study shows that there is good correlation between morphine concentration in the blood and that in the brain, particularly in those areas of the brain which have high concentrations of opiate receptors, the thalamus and brain stem.

It may seem surprising that correlation in the cerebral cortex is so low. This may be due to the fact that, because of the large size of the cerebrum, the samples analyzed came from various areas from within the cerebrum. Samples labeled frontal lobe, occipital lobe, cerebral cortex and basal ganglia were all placed in the same category for purposes of this study.

As a result of this study it can be concluded that the
level of morphine in the blood can be used to predict the level in the brain, the best correlation being between the thalamus and blood.
APPENDIX

CONFIRMATION OF DERIVATIZATION

The chromatogram was obtained on a Finnigan 4000 GC-MS using a 30m SE54, fused silica capillary column with a 0.25 mm. I.D. and a 1μl sample.

- Initial temperature 150°C Initial time 5 min
- Final temperature 310°C Ramp time 5°/min
- Carrier gas, helium 1 ml/min

The peaks were identified by spectroscopists at the Muskegon County Wastewater Laboratory. Peaks a, c and d are phthalates, common contaminants extracted from plastic. Peak b is the trimethylsilyl derivative of a fatty acid. Peak e is bis(trimethylsilyl)morphine.

The mass spectrum was started 9 min after the sample injection. An electron impact source was used. The most intense peaks and their relative abundance are given below.

<table>
<thead>
<tr>
<th>Mass</th>
<th>% Relative abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>73</td>
<td>100</td>
</tr>
<tr>
<td>236</td>
<td>61</td>
</tr>
<tr>
<td>146</td>
<td>32</td>
</tr>
<tr>
<td>234</td>
<td>30</td>
</tr>
<tr>
<td>220</td>
<td>25</td>
</tr>
<tr>
<td>237</td>
<td>21</td>
</tr>
<tr>
<td>429</td>
<td>16</td>
</tr>
<tr>
<td>287</td>
<td>13</td>
</tr>
<tr>
<td>75</td>
<td>10</td>
</tr>
</tbody>
</table>
BIBLIOGRAPHY


95


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Patents with D. W. Young
U.S. #3,031,495
3,062,754
3,150,091
3,202,701
French #1,227,255

102