Chapter I

Drug Identification and characterization.

Identification and differentiation of drugs have been the most common approach for forensic toxicologist, a successfully designed experiment for identification and differentiation of a drug should give satisfaction to help in solving a cause of death resulting from drug over-dose. Further more, good selection of different organs and determine which organ and/or organs that contain the highest concentration of the parent drug.

Different toxicological analytical techniques were applied to reveal the various factors, which may interfere with the characterization of the drug. These techniques were color test, thin layer chromatography (TLC), ultraviolet spectroscopy (UV), high performance liquid chromatography (HPLC), infrared spectroscopy (IR), proton nuclear magnetic resonance (^{1}H- NMR) and Mass spectra (MS).

The present work revealed the following results:

I- Spot test:

The first step in the experiment design is the color test. Although color test is simple and was carried out for the presumptive identification of the drug, it can help in saving time and coast and narrow windows of search. Spot test is not specific as it depends on variation of colors with different drugs and reagents used since spot tests usually screen for a class or a broad category of compounds, positive results are usually considered to be presumptive, Steinert and Coffman (1992).

In spite of its permittivity its results combined with other identification techniques it can be interpreted as definitive proof of the identification of different drugs.

Table (1) illustrates the different colors obtained by nalbuphine using different chemical reagents from which the following observations can deduced.

Table (1): Colors produced by nalbuphine using different chemical reagents

REAGENT	Nalbuphine
Beam’s	-Ve
Chen-kao	-Ve
Cobalt nitrate/Ammonia	Blue
Cobalt thiocyanate	-Ve
Conc. Sulphuric acid	-Ve
Ferric chloride	-Ve
F P N	-Ve
Forrest’s	-Ve
Frohde’s	Red
Furfural	-Ve
Liebermann's	-Ve
Mandelin’s	-Ve
Marqui’s	Violet
P D A	Pale yellow
Potassium permanganate	Pale brown
Potassium permanganate/NaoH	Pale brown
Sodium hydroxide	-Ve
Vitali - Morrine	-Ve

Zwikker	-Ve

Nalbuphine does not give any color with Beam's reagent, Chen-Kao, cobalt thiocyanate, conc. sulfuric acid, ferric chloride, FBN, Forrest's, furfural, Lieberman's, Mandolin's, Vitali Morin and Zwikker

reagents. These results mean that these reagents are not suitable for the identification of nalbuphine.

On the other hand, different colors were found to be characteristic for nalbuphine as follow:

* Blue color with Cobalt nitrate/ Ammonia reagent. Also, pale yellow color was obtained with PDA reagent.

* Violet color was produced by Marquis Reagent. While red color was produced by Frohde's reagent. These results could be explained as these two reagents act on aromatic compounds which consist entirely of C, H and N, Clarke (2004).

* Pale brown color was produced by both potassium permanganate and potassium permanganate/NaOH reagents, indicating that the drug has the ability to produce a typical reduction reaction.

However, the color tests are not specific, a combination of thin layer Chromatography (TLC) and color reactions have been used to identify various drugs, Mule (1971).

II) Thin layer chromatography (TLC):-

Visualization of spots of the drugs on the chromatoplates under uv lamp and/or by using different coloring reagents helps in locating the

spots on the plates after elution by different eluting solvents. Burtis et al., (1987) reported that spots of compounds on the TLC chromatograms are usually identified by their R_F values. However, Spratt (1977) Demonstrated that the R_F value of a given drug may vary according to humidity, temperature and size of the chamber used.

Table (2): illustrate the colors obtained by nulbuphin under uv lamp and/or after spraying with different coloring reagents.

Table (2): Colors obtained by the nalbuphine under uv lamp and/or after using different chemical spraying reagents

Spraying color reagents	nalbuphine
1- Examination under UV lamp	Blue

using silica gel GF254	
2- Mineral acids:	
a) Nitric acid 2%	-ve
b) Hydrochloric acid 2%	-ve
c) Sulphuric acid 2%	-ve
3- Reagent that are reduced with organic compounds:	
- Ferric chloride 5%	-ve
4- General reagents for basic Drugs:	
a) Dragendorff	Orange
b) Iodoplatinate	Violet
5- Aldehyde:	
a) Vanillin / sulfuric acid	-ve
b) Furfuraldehyde / sulfuric acid	-ve
6- Miscellaneous:	
a) PDA	pale yellow
b) FPN	-ve
c) Alcoholic sodium hydroxide	-ve
d) Potassium permanganate	pale brown
e) Zwikker	-ve
f) Paladus chloride	-ve
g) Iodine vapour	Brown

Nalbuphine showed blue color under short UV lamp. Different coloring reagents were then sprayed on the chromatoplates as direct coloring reagents. Spraying the chromatoplates by some mineral acid

reagents, that is reduced with organic compounds. Aldehyde reagents and some miscellaneous reagents as FPN, PDA, Zwikker, and Beams reagents and paladus chloride give no color with nalbuphine. Dragendorff reagent, iodine vapour and potassium permanganate produced orange, brown, pale brown colors respectively with nalbuphine.

Mean while these reagent give positive reaction with primary, secondary, tertiary and quaternary amines, Clarke (2004). So these reagents couldn't be considered as specific spraying reagents for nalbuphine.

Iodoplatinate reagent produced violet color with nalbuphine it could be considered as specific reagent to identify or differentiate the studied drug.

Calculated R_F values for nalbuphine:-

The main target aimed at the thin layer chromatographic studies of the drug was the separation and identification of the studied drug. The chromatographic behavior of nalbuphine on silica get GF_{254} is considered important for its identification. Different eluting solvents of moderate polarity were used in the present work with a considerable R_F values Table (3).

Fig. (1) Illustrate the chromatoplates of nalbuphine using different eluents and sprayed with iodoplatinate reagents.

The present study revealed that nalbuphine did not need eluting solvents of high polarity. As concluded, the best eluents were isopropyl alcohol: chloroform: ammonia (90:90:2) (R_f = 0.78), methanol (R_f = 0.69), acetone (R_f = 0.65) methanol: ammonia (100: 1.5) (R_f = 0.78), benzene: ethanol (7:3) (R_f = 0.52).

Table (3) Calculated R_F values for nalbuphine using different eluents

Eluents	Nalbuphine

(E1) Toluene:Acetone :Ethylacetate: Ammonia (10 : 10 : 3 : 0.75)	0.39
(E2) Chloroform: Methanol (4:1)	0.95
(E3) Ethyl acetate : Methanol : Ammonia (85 : 10 : 5)	0.58
(E4) Methanol (100)	0.69
(E5) Methanol:Ammonia(100:1.5)	0.78
(E6) Benzene : Ethanol (7 : 3)	0.52
(E7) Chloroform : Methanol (9 : 1)	0.22
(E8) Isopropyl alcohol : Chloroform : Ammonia (90 : 90 : 20)	0.78
(E9) Cyclohexane : Toluene: Diethyleamine (75 : 15 : 10)	0.08
(E10) Acetone (100%)	0.66

III) High Performance Liquid Chromatography (HPLC):-

High performance liquid chromatography (HPLC) determination of drugs extracts is of reliable and definitive method for identification and quantification of drugs in different samples.

In the present work retention time (R_t) was determined for nalbuphine, it was 3.33 min. which was obtained by using ultraviolet spectrophotometer (λ max. =254 nm). However, Quarry et al., (1998) detected nalbuphine by using HPLC connected with UV spectrophotometer at wave lengths λmax. =280. Also, different investigators determined nalbuphine by using HPLC with different eluting solvents depending on the polarity of the eluting solvents used (Kuhnert et al., 1983;Keegan et al., 1984; Lo et al., 1984; Dube et al., 1988; Wetzelsberger et al., 1988; Ho et al., 1991; Borg et al.,1993;Chou et al., 1993; Nicolle et al., 1995; Ho et al., 1996; De- Casanova et al., 1997; Liaw et al., 1998; Quarry et al., 1998; Pao et al., 2000; Groenendaal et al., 2005; Heng pao et al., 2005; Fang et al., 2005; Leung et al., 2005).

Acetonitrile / methanol (1:1) were used as eluent and the column was BDS C18.

The results obtained in this study were considerably convenient especially with the use of a new trend of reversed phase columns (BDC). Also the use of mixed acetonitrile /methanol (1:1) eluent with a flow rate of 1 ml /min. proved to be highly convenient for separation of nalbuphine from different biological samples examined. Moreover, this method proved to be valuable due to saving solvent and time.

Figure (2) shows HPLC chromatograms of nalbuphine and figure (3) shows nalbuphine calibration curve.

Fig. (2) Nalbuphine HPLC chromatogram

Fig. (3) Nalbuphine calibration curve obtained using HPLC

IV) Ultraviolet spectroscopy:-

Ultraviolet spectrophotometric examination of drugs provides a simple and considerably reliable means of identification of pure drugs.

Steinert and Coffman (1992) reported that the utility of spectrophotometry comes from the fact that each substance has its own absorption spectrum; therefore it can be used for its identification.

The present study revealed that nalbuphine showed maximum absorbance at λ_{max}= 248 (fig.4).

However, Quarry et al., (1998) measured nalbuphine at λ_{max} 280 nm.

The difference in the values of λ_{max} may be due to the polarity of the eluents used.

Fig. (4) Nalbuphine UV spectrum

v) Infrared spectroscopy:-

Examination of nalbuphine hydrochloride by using infrared spectrophotometry revealed that it may be identified through an equivalence of the absorption spectrum of the analyte with the characteristic infrared absorption spectrum (KBr pellet method) showed band at ע 3430-3415 cm^{-1} due to oH ,at 3259 cm^{-1} due to NH+.

VI)Hydrogen nuclear magnetic resonance (^{1}H- NMR):-

The ^{1}H -NMR spectrum of nalbuphine dissolved in chloroform showed the corresponding spectral assignments as follow: 1.20(m,16H,CH2) ,1.67(m,3H,CH),3.2(m,4H,NCH2),3.83(S,H,NH+),5.22(S,2H,OH), 7.63(m,2H,Ar-H),10.10(S,H,OH).

VII) Mass spectroscopy (MS):-

The characteristic mass spectrum of nalbuphine showed molecular ion peak at m/z=358(see fig. 5) which is in agreement with that reported by Andruas et al., 1987; Fang et al., 1989; Xu et al., 1991; Kintz et al., 1992; Yoo et al., 1995; Moore et al., 1997; Andraus et al., 1998; Kim et al., 2004; Leung et al., 2005.

Fig. (5) Mass spectrum of nalbuphine

In the present study, the results of application of different techniques for the analysis such as IR spectroscopy, ^{1}H-NMR, Mass spectroscopy helped in identifying nalbuphine in its pure form.

It was concluded that combination of different techniques of analysis can help in identifying and determination of nalbuphine in its pure form.

Chapter II

Detection of nalbuphine in post mortem rat tissues

Present technologies have brought a tremendous development of diversified drugs and chemicals. So it became absolutely necessary to monitor drugs. atoxic effects or outright poisoning have as a result, became an every day happening all over the world. In suspected drug intoxication, one must determine if the amount of toxicant present in the appropriate specimens is consistent with producing lethality. Therefore, detection and evaluating concentration of the drug in postmortem tissues, provides valuable information regarding the cause and manner of death.

Nalbuphine is a synthetic opioid analgesic which is chemically derived from the oxymorphone; it is used as alternative drug for different addicts. The need for a procedure to detect nalbuphine in biological samples is becoming increasingly important.

In the present study ammonium sulphate methode, Nickolls (1956) was used for the extraction of nalbuphine from different tissues and blood. This method has been found to be more convenient procedure for

precipitation of protein and consequently extraction of the drugs in wide range of concentration from the tissues. However ,several investigators previously used different methods for extraction of the drugs from the blood and urine samples other than extraction by chloroform and using ammonium sulphate method for precipitation of proteins to the extraction of nalbuphine from tissue samples (Kuhnert et al., 1983; Keegan et al., 1984; Lo et al., 1984; Kintz et al.,1992; Brog et al., 1993; Chou et al., 1993; Nicolle et al., 1996; Yoo et al., 1995; Ho et al., 1996; Pao et al., 2000; Kim et al., 2004; Groenendaal et al.,2005; and Heng pao et al., 2005).

In the present study, the application of HPLC method (with UV detector and acetonitrile/methanol (1:1) as mobile phase) for quantitative evaluation of nalbuphine concentration in biological matrix was generally in reasonable agreement with previous experiments, confirming the advantages of such techniques in toxicological investigations. Moreover, the use of isocratic method has the advantage of low bubbles production and low cost.

A comprehensive approach to the analysis for the nalbuphine in postmortem tissues and biological fluids using HPLC has been developed (Kuhnert et al 1983; Keegan et al., 1984; Lo et al., 1984; Dube et al., 1988; Wetzelzberger et al., 1988; Ho et al. ,1991; Brog et al., 1993; Chou

et al.,1993; Nicolle et al., 1995; Ho et al., 1996; De-Cazanove et al., 1997; Liaw et al., 1998; Quarry et al., 1998; Pao et al., 2000; Groenendaal et al., 2005; Heng pao et al., 2005; Fang et al., 2005). Also, Leung et al., (2005) developed a sensitive method for analysis of nalbuphine using HPLC tandom mass spectrometry (LC-MS-MS). Therefore these investigations suggested that HPLC method for analysis is sensitive, reliable and reproducible techniques for toxicological analysis of nalbuphine.

Table (4) shows the concentration of nalbuphine in tissues obtained from rats administrated single dose (10 mg/kg body weight) and sacrificed after four hours of treatment using HPLC with UV detector.

Table (4) Nalbuphine concentrations (ng/g rat tissue) in samples obtained from rats administrated a dose of 10 mg/ kg b. wt. and sacrificed after 4 hours of Treatment.

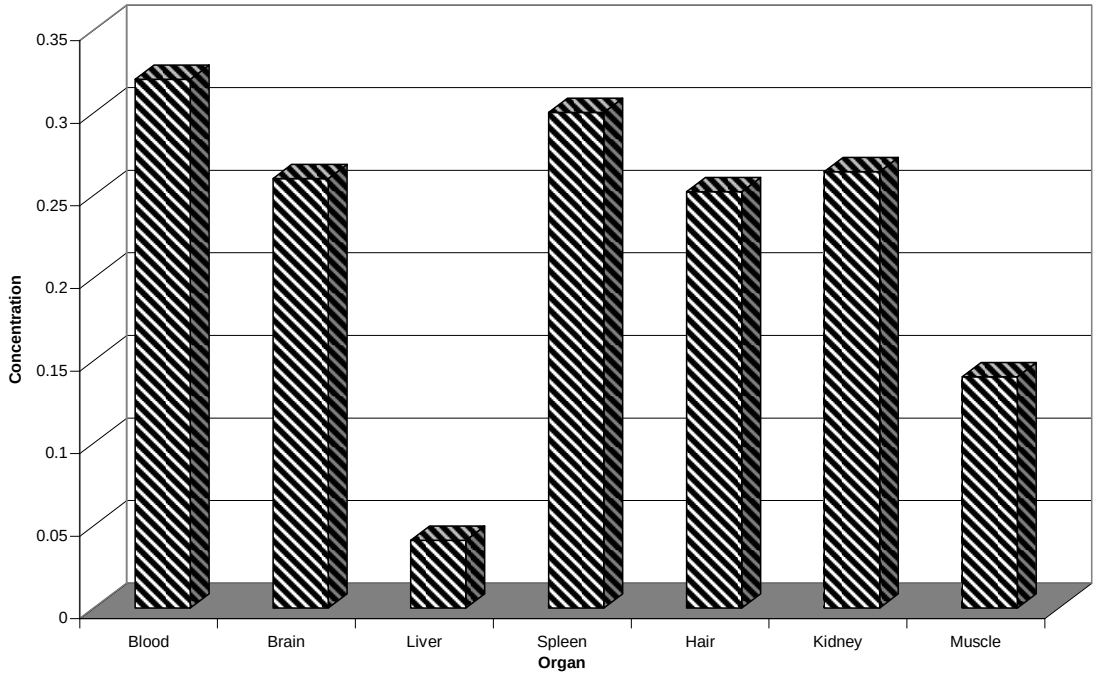
Animal No.	Blood	Brain	Liver	Spleen	Hair	Kidney	Muscle
1	0.2700	0.3100	0.0390	0.4300	0.2400	0.1500	0.1100
2	0.2400	0.1800	0.0230	0.3200	0.4100	0.2500	0.1300
3	0.3000	0.2100	0.0320	0.2000	0.1800	0.2900	0.1800
4	0.3800	0.3800	0.0590	0.3000	0.3200	0.2800	0.2200
5	0.4100	0.2200	0.0520	0.2500	0.1100	0.3500	0.0600
Mean	0.3200	0.2600	0.0410	0.3000	0.2520	0.2640	0.1400

Standard Error	**ó± 0.0725**	**±0.0029**	**± 0.0146**	**± 0.0863**	**± 0.1173**	**± 0.0733**	**± 0.0620**
Standard deviation	**± 0.0145**	**±0.0006**	**± 0.0029**	**± 0.0173**	**± 0.0235**	**± 0.0147**	**±0.0124**

Nalbuphine was detected with mean concentrations of 0.32±0.0145, 0.30 ±0.0173, 0.041 ± 0.0029, 0.260± 0.026, 0.25 ±0.0235, 0.26±0.0147 and 0.14 ±0.0124 ng/g tissues in blood, spleen, liver, brain hair, kidney and muscle sample tissues respectively

Fig. (6) Shows the concentration of nalbuphine in tissues obtained from rats administrated a dose of 10 mg / kg body weight.

Fig. (6) Distribution of nalbuphine in different rat organs in a dose of 10 mg/kg rat tissues.


0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Concentration
Blood
Brain
Liver
Spleen
Hair
Kidney
Muscle
Organ

This figure illustrates the following:

Nalbuphine was in different tissue with a gradient in concentration from maximum to minimum blood, spleen, brain, kidney, hair, muscle and liver (fig. 6).

Table (5) shows the concentration of nalbuphine in tissues obtained from rats administrated single dose (20 mg/kg body weight) and scarified after four hours of treatment.

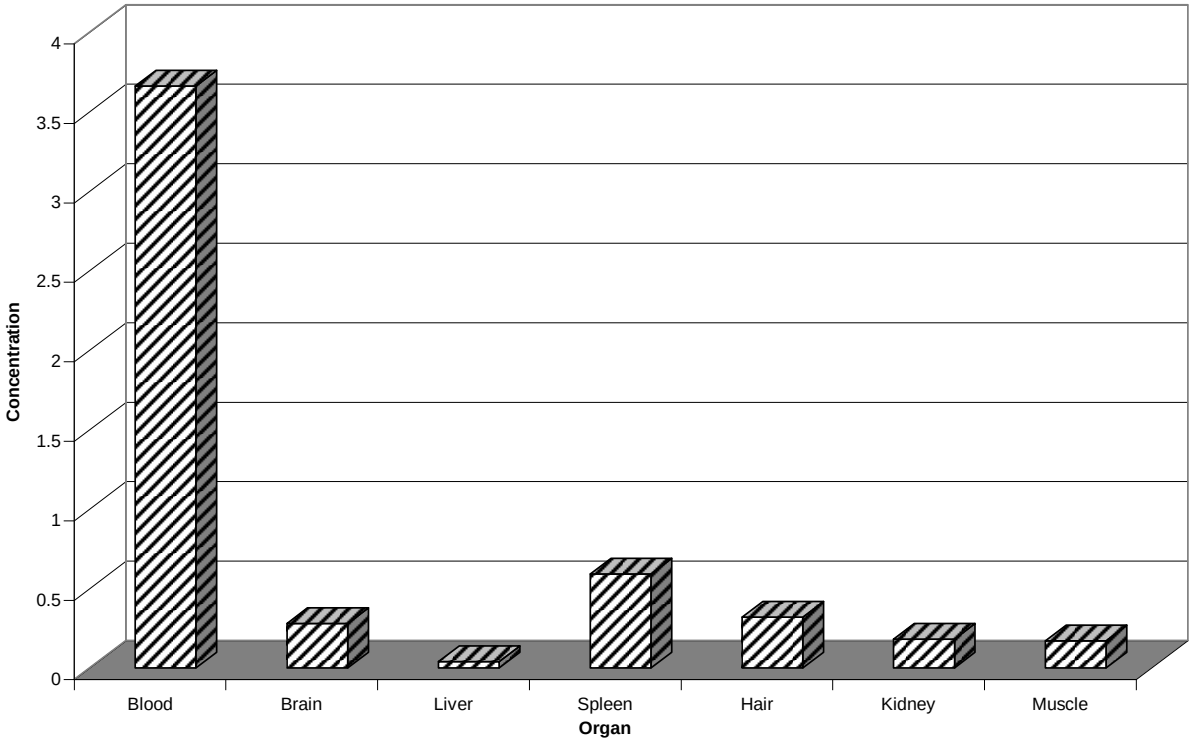
Table (5) Nalbuphine concentrations (ng/g rat tissue) in samples obtained from rats administrated a dose of 20 mg/ kg b. wt. and sacrificed after 4 hours of Treatment

Animal No.	Blood	Brain	Liver	Spleen	Hair	Kidney	Muscle
1	3.6700	0.2500	0.0100	0.5800	0.2200	0.2300	0.1300
2	2.9900	0.2300	0.0700	0.5100	0.3100	0.1600	0.1700
3	4.3300	0.3600	0.0300	0.6900	0.2400	0.1000	0.1600
4	5.2300	0.1200	0.0600	0.7900	0.4500	0.2600	0.2300
5	2.0900	0.4400	0.0200	0.5900	0.4100	0.1500	0.1900
Mean	3.6620	0.2800	0.0380	0.5925	0.3260	0.1800	0.1760
Standard Error	± 1.2070	±0.1235	±0.0259	±0.0741	±0.1016	±0.0644	±0.0371
Standard deviation	± 0.2414	±0.0247	±0.0052	±0.0148	±0.0203	±0.0129	±0.0074

Nalbuphine was detected with a mean concentrations of 3.662± 0.2414, 0.28± 0.0247, 0.038± 0.0052, 0.59± o.o148, 0.326± 0.0203, 0.18± 0.0129 and 0.176± 0.0074 ng/g in blood ,brain ,liver ,spleen hair ,kidney and muscle tissue samples respectively.

Fig. (7) Shows the concentration of Nalbuphine in tissues obtained from rats administrated a dose of 20 mg/kg body weight.

Fig. (7) Distribution of nalbuphine in different rat organs in a dose of 20 mg/kg rat tissues.



This figure illustrates the following:

Nalbuphine was in different tissue with a gradient in concentration from maximum to minimum blood, spleen, hair, brain, kidney, muscle and liver, fig. (7).

From the results of the present study the following observations could be shown:

1- Blood had the highest concentration of nalbuphine at different dose levels used in this study. This finding may be explained according to Stadnyk and Grossman (1987) who reported that plasma half life of nalbuphine is five hours following a single intravenous dose. Also, Martin Dale (1999) mentioned that nalbuphine has been reported to produce peak plasma concentration after 30 minutes.

2- Concentration of nalbuphine in spleen was as high as the concentration of it in blood. This finding can be explained according to Casarett and Doull (1996) who stated that spleen tissue has high content of blood as it is the organ responsible for the blood filtration.

3- Concentration of nalbuphine in brain tissue was rather high especially

At different doses especially at the lower dose of nalbuphine (10 mg/kg body weight) this can be explained according to the lipophilicity of the drug (Clarke 2004). Casarett and Doull (1996) studied that the blood-brain barrier permits the lipophilic drugs to cross it simply.

4 –Concentration of nalbuphine in kidney tissue was moderate this could be explained according to Clarke (2004) who reported that unchanged nalbuphine and its conjugates have been detected in urine. Also, Micromedix data base (2007) reported that nalbnphine is eliminated in urine (about 7%) unchanged. Meanwhile, Martine Dale (1999) mentioned that nalbuphine is excreted predominantly in the faeces as unchanged drug and conjugate with other melabolic products, about 7% of a dose has been reported to be excreted in the urine as unchanged drug.

5- Concentration of Nalbuphine in hair was rather near in both low and high doses; this finding may be explained according to Baumgartner et al (1989) who repeated that drug accumulation in hair is roughly proportional to the amount of drug injected. The link between drugs used and drug accumulation in hair has been examined in several earlier reports (püschel et al., 1983; Uematsu et al., 1989; Miyazawara et al, 1991 and kintz and mangine, 1992). It is possible that drugs are incorporated into hair via a more complex way in which drugs are

transferred from the body to hair from multiple pool, and during various times in hairs life cycle, Henderson (1993). So, in this study it was revealed that hair can be used as samples of choice in forensic science because it is easy to obtain and store, traqui et al., (1995). Several factors were found influencing the accumulation of chemicals in the hair matrix. Cone et al., (1993) mentioned that there is a correlation between the amount of opiates present in beard hair and approximate time of dosing. Nakahara et al., (1995) hypothesized that the basically and lipophilicity strongly affected the increase of incorporation rate of drugs into hair. Again, several reports suggested that the hair pigmentation has some influence on the drug concentration in hair samples, since the measured drugs concentrations (haloperidol, ofloxacine, p.bromo methyl amphetamine and methadone) were found higher in pigmented than in non pigmented hair (bathory et al., 1990; Uematoso et al., 1990; umatsu et al., 1992 and Wilson et al., 1995). Meanwhile, püschel et al., (1983) pointed out that the growth rate of the hair, diffusion and adhesion processes may influence the transport of drugs along the hair.

6- The concentration of nalbuphine in muscle indicated that muscle tissue can be considered to be the sample of choice in case of absence of blood samples. Christensen et al., (1985) suggested that the skeletal muscle should be included as standard sample in examinations for drug

poisoning. The present findings may be confirming the importance of such tissue in the evaluation of fatal cases of drug overdose, Garriot (1991). In addition, Pawnder et al., (1996) recommended that skeletal muscle must be sampled from a limb. Also, Ito et al., (1997) mentioned that the skeletal muscle is a sample which can be obtained from a cadaver which has been buried under ground for along period of time and thus is considered to be a suitable sample for toxicological examinations.

7- Liver tissue was considered to have the lowest concentration of nalbuphine, this could be explained by its extensive metabolism. Martine Dale (1999) reported that nalbuphine metabolism is meanly occurred in liver. Jacqz-aigrain et al., (2003) administered nalbuphine intravenously in critically ill neonates under going invasive procedures differences in the maturation of hepatic and renal function affect the disposition of drugs in neonates as nalbuphine is primarily eliminated by metabolism, they found that clearance of nalbuphine was lower than the values reported in older infants, children and adults. Also Clarke (2004) reported that nalbuphine major metabolic reaction is conjugation to from nalbuphine glucouronide (inactive); oxidation of the later gave 6-oxonalbuphine occur, in addition to the desalkylated derivatives 7, 8 dihydro 14hydroxy normorphine, and hydroxy normorphine has also been identified as a metabolite.

Further more, the root of administration of a drug can differ in its way of distribution. Lukas et al, (1997) mentioned that intraperitoneal administration of compounds are absorbed primarily from the portal circulation and therefore, must pass through the liver before reaching other organs where 1^{st} path metabolism takes place.