The aim of this study was to examine the regional distribution of opiate alkaloids from seized heroin in experimental animals' brain regions and blood. Results could be used in the examination of opiate alkaloids' distribution in human biological samples in order to contribute to the solution of the causes of death due to heroin intake.

Experimental animals (Wistar rats) were treated with seized heroin, and were sacrificed at different time periods: 5, 15, 45 and 120 min after treatment.

Opiate alkaloids' (codeine, morphine, acetylcodeine, 6-acetylmorphine and 3,6-diacetylmorphine) content was determined in the brain regions (cortex, brainstem, amygdala and basal ganglia) and blood of animals using gas chromatography–mass spectrometry (GC–MS) method.

The highest content of opiate alkaloids in the blood was measured 15 min, and in the brain tissue 45 min after the treatment with heroin. The maximal concentration of opiates was determined in the basal ganglia.

The obtained results offer the possibility of selecting this part of the brain tissue as a representative sample for identifying and assessing the content of opiates.

Key words: brain tissue, experimental animals, GC-MS, heroin, opiates' distribution

INTRODUCTION

Opiate alkaloids (opiates) are narcotic analgetics, belong to the group of natural or synthetic compounds that exhibit an activity similar to morphine, the main agent for the alleviation of pain. Of all the alkaloids present in opium, morphine takes first place, both in terms of relative presence, as well as the therapeutic significance and toxicity (Rogers et al., 1981).
Analgesia is the most important pharmacological effect of morphine, which is the reason for its clinical use. Although the central nervous system (CNS) is the primary site of action of morphine, unlike heroin and codeine, only 20% of the ingested dose of morphine can pass the blood-brain barrier and reach the brain, due to transport by P-glycoprotein. This barrier to morphine does not exist in children, which is one of the reasons for their special sensitivity to morphine (Letrent and Polli, 1999). The major disadvantage of morphine application for medical purposes is that prolonged therapy leads to tolerance, psychic and physical dependence.

Heroin (3,6-diacetylmorphine) is a semisynthetic derivative of morphine. It is more toxic than morphine, has a three fold stronger effect and very quickly leads to addiction. It is assumed that heroin is the most addictive drug. Today, heroin is almost exclusively abused, which causes immense health problems and consequences.

In Serbia’s illegal market, heroin powder is of light brown color, and except 3,6-diacetyl morphine (5-50%), usually contains opiate alkaloids: meconin, hydrocotamine, thebaol, acetylthebaol, codeine, acetycodeine, morphine, monoacetylmorphine (3- and 6-acetylmorphine), papaverine and noscapine. In order to increase its market weight, various additives are added to heroin: sugar (glucose, sucrose, lactose, mannitol, dextrose), starch, quinine, paracetamol, caffeine, procaine, diphenhydramine.

Heroin is a drug that one never knows what percentage of pure substance 3,6-diacetylmorphine it contains. It often happens that, if "lower percentage" heroin is consumed, then unknowingly "higher percentage" heroin overdose may occur immediately after its application, which can be fatal.

However, often a relatively small amount of heroin can cause death due to cardiovascular collapse and pulmonary edema. This syndrome is known as acute fatal reaction, or syndrome X, which most commonly occurs if heroin is consumed with CNS depressants such as alcohol and barbiturates.

Morphine and its related compounds bind to specific sites in the body and cause pharmacological effects. The opiate receptors, are primarily located in the brain and spinal cord, on the neuronal cell membranes.

Since opiate receptors have been cloned and their molecular structures described, pharmacological studies confirmed that the most important pharmacological effects of opiate alkaloids occur through three major classes of opiate receptors, μ (mu), κ (kappa) and δ (delta), each containing several subclasses (Aggrawal, 1995; Dhawan et al., 1996).

Toxicity of opiates is directly caused by activation, i.e. binding to μ-receptors, which play an important role in pain and analgesia, respiratory and cardiovascular functions, peristalsis, nutrition, mood, thermoregulation, hormone secretion and regulation of immune functions (Viganó, 2003).

Immunohistochemical studies of human brain in deaths which are due to excessive doses of morphine intake showed that morphine localizes in the cytoplasm of neurons of the cortex, basal ganglia, brainstem, cerebellum, and part of the limbic system - hippocampus and amygdala (Atweh and Kuhar, 1997a, 1997b, 1997c).
Numerous in vivo and in vitro experiments were carried out to determine whether changes in the density of μ-opiate receptors is responsible for tolerance, dependence and a wide range of concentrations of morphine determined in certain cases of fatal intoxication (Kaa and Teige, 1993; Skopp et al., 1996). In some cases, for example long-term application of morphine to guinea pigs and rats, showed a decrease in μ-opiate receptor density (Tao, 1990). It is assumed that μ-receptor density significantly decreased with age, which is associated with reduced sensitivity to opiate agonists (Jha, 2004). In contrast, previous experiments with mice (Rothman et al., 1986) and quantitative autoradiography analysis of rats brain (Brady et al., 1989) have shown an increase in μ-opiate receptors density after long-term application of morphine. Comparing the effects of morphine and heroin on the rats brain, increasing in the number of μ-opiate receptors was confirmed in the group of animals dependent on heroin, but not in the groups of animals dependent on morphine (Bolger et al., 1988).

Immunohistochemical studies of human brain (Schmidt et al., 2001) are in accordance with previous experiments of ligands binding (Brunello et al., 1984), autoradiographic analysis (Geary and Wooten, 1985) and in vitro experiments on cell cultures, where changes in μ-opiate receptors density were not proven after long-term application of morphine. It could be said that a long-term application of exogenous opiates causes the receptors to change their conformation and thus reduce in sensitivity, thus requiring higher doses of opiates to achieve the same effect (Rogers and El-Fakahany, 1986).

In contrast to the opiate agonist, studies on the influence of opiate antagonists on the regulation of μ-opiate receptors showed identical results. Long-term usage of opiate antagonists naloxone and naltrexone caused "hypersensitivity" of opiate receptors against agonists and an increase in their density (Brodsky et al., 1995).

The aim of this study was to examine the regional distribution of opiate alkaloids from seized heroin: codeine, morphine, acetylcodine, 6-acetylmorphine and 3,6-diacetylmorphine in the following parts of the brain (known for their high density of μ-opiate receptors): cortex, brainstem, amygdala and basal ganglia, and blood of experimental animals at different periods of heroin treatment of animals. The obtained results should facilitate the selection of parts of brain tissue with the highest content of opiates and by analysis of biological samples of human brain tissue, would contribute to resolve the causes of death due to heroin abuse.

MATERIAL AND METHODS

Heroin

Heroin for treating experimental animals, obtained from police authorities, was seized in Novi Sad during 2010.

In order to determine the qualitative composition and amount of the most abundant opiate alkaloids (6-acetylmorphine and 3,6-diacetylmorphine), to the seized heroin (5 mg) a solution of the internal standard (IS) of meperidine (0.5 mL;
3.0 µg/mL) and 0.5 mL of alkaline distilled water (pH 9, adjusted by adding solid buffer $K_2CO_3$-$NaHCO_3=2:3$ and 25% $NH_4OH$) were added. Extraction was carried out with ethyl acetate (1 mL) for 2 minutes on a Vortex T-Genie 2 apparatus. After centrifuging at 3000 rpm for 5 min, anhydrous $Na_2SO_4$ was added to the organic layer which was then evaporated to dryness under a gentle stream of nitrogen at 40°C, reconstituted in 0.5 mL of methylene chloride and 1 µL was injected into the gas chromatograph with mass spectrometer (GC–MS).

By qualitative GC-MS analysis was determined that the seized heroin contains a mixture of opiate alkaloids: meconin, hydrocotarnine, thebaol, acetylcodine, 6-acetylmorphine, 3,6-diacyethylmorphine, papaverine, noscapine, and paracetamol and caffeine as additives. Quantitative GC-MS analysis showed that the analyzed sample contains 9.62 % of 3,6-diacyethylmorphine and 8.38 % of 6-acetylmorphine.

Based on the content of the most abundant psychoactive opiate alkaloids in the mixture, the optimal dose of heroin was prepared for the experimental treatment.

For the study heroin was prepared so to imitate most closely the way it is used by heroin addicts. To seized heroin (0.56 g) citric acid (0.11 g) and physiological solution (0.9% $NaCl$; 5 mL) were added, and the mixture was heated to boiling. The dark-brown solution was then filtered and diluted with saline to the final volume of 10 mL. The concentration of such a solution was 10 mg/mL (calculated on the content of 3,6-diacyethylmorphine and 6-acetylmorphine in the mixture).

**Animals**

Experiments were carried out on white six-month old male Wistar rats, body weight 280–350 g. The animals were selected by random choice from the 2010 litter of the Department of Biology, Faculty of Sciences in Novi Sad. Until the beginning of the experiment, rats were kept in separate cages in a climatized room at a temperature of 20–25°C, exposed to a 12-hour succession of light and dark periods, and had free access to food and water.

All the experiments and protocols employed in the study were reviewed and approved by the Institutional Animal Care and Use Committee.

The animals were divided into two groups:

1. control group (10 animals) – upon weighing, were treated with physiological solution (about 1 mL) and sacrificed after 15 minutes, and
2. experimental group (20 animals) – upon weighing received 25 mg/kg b.w. (about 1 mL) of the prepared heroin solution (10 mg/mL), and sacrificed after: 5, 15, 45 and 120 minutes, each time 5 animals.

Heroin was administered intraperitoneally with the aid of an insulin syringe (1 mL), and after treatment the animals were kept in separate cages. The animals were sacrificed by decapitation. After decapitation, blood was drawn from the heart and mixed with sodium fluoride to prevent decomposition of drugs. The brain was quickly removed, rinsed with distilled water and the brain regions (cortex, brainstem, amygdala and basal ganglia) were collected. Samples were stored at −80°C until drug analysis.
Preparation of biological samples

To the blood samples (0.5 - 1 mL) the solution of the internal standard (IS) of meperidine (0.2 mL; 3.0 μg/mL), and solid buffer K₂CO₃:NaHCO₃=2:3 and 25% NH₄OH were added to attain pH 9, and the content was diluted to 5 mL with distilled water.

Samples of cortex, brainstem, amygdala and basal ganglia (0.1–0.25 g) were homogenized in a mortar with quartz sand (0.5 g) and sodium chloride (0.5 g). The internal standard (0.2 mL; 3.0 μg/mL), and 5 mL of acidified distilled water (pH 2, adjusted by adding 2M H₂SO₄) were added to the brain homogenate solution. Extraction was carried out with n-hexane (3 mL) to remove higher fatty acids and cholesterol for 2 minutes on a Vortex T-Genie 2 apparatus. After centrifuging at 3000 rpm for 10 min the organic layer was discarded, while the solid buffer K₂CO₃:NaHCO₃=2:3 and 25% NH₄OH were added to the aqueous layer to adjust to pH 9.

Diluted blood samples and brain homogenates were extracted by solid phase extraction (SPE) using AccuBOND EVIDEX II extraction columns (Agilent Technologies, USA). The columns were preconditioned with 5 mL of methanol and with 8 mL phosphate buffer pH 6 (0.1 M K₂HPO₄: 1.74 g anhydrous K₂HPO₄ were dissolved in 100 mL of distilled water and adjusted to pH 6 with cc H₃PO₄). The diluted and homogenized samples were loaded on the columns and aspirated by vacuum at a flow rate of 5 mL/min. The columns were washed with 3 mL of distilled water and 3 mL of acetate buffer pH 4.5 (0.1 M CH₃COONa:0.82 g of CH₃COONa was dissolved in 100 mL of distilled water and adjusted to pH 4.5 with ice acetic acid), and then dried for 10 min. The analyte was eluated with 6 mL of the mixture of solvents methylene chloride:iso-propanol:ammonium hydroxide = 78:20:2. The eluates were evaporated to dryness under a gentle stream of nitrogen at 40°C, reconstituted in 0.2 mL of methylene chloride and 1 μL was injected into the GC–MS.

Gas chromatography-mass spectrometry analysis

Agilent 6890 N gas chromatograph (GC) equipped with Agilent 5973 mass selective detector (MSD), Agilent autosampler 7683 and Agilent DB–5MS capillary column (30 m, 0.25 i.d., 0.25 μm film thickness) was used. The MS detector was operated in electron impact (EI) mode at 70 eV with interface temperature of 280°C. The injection port temperature was 250°C. GC was performed in splitless mode, carrier gas was helium at a constant flow rate of 1 mL/min. The column temperature was programmed as follows: an initial temperature of 50°C increased to 200°C at a rate of 40°C/min, then to 280°C at a rate of 20°C/min, and was maintained for 12.25 min.

Selected ion monitoring (SIM) was performed at m/z 299, 229 and 162 for codeine, at m/z 285, 162 and 215 for morphine, at m/z 341, 282 and 229 for acetylcocodeine, at m/z 327, 268 and 215 for 6-acetylmorphine, at m/z 369, 327 and 268 for 3,6-diacetylmorphine and at m/z 247, 172 and 218 for internal standard of meperidine.

Separate stock solutions of opiate alkaloids and internal standard (meperidine) were prepared at a concentration of 100 μg/mL in distilled water by
diluting the original standard solutions (in methanol) of codeine (1 mg/mL), morphine (1 mg/mL), acetylcodine (1 mg/mL), 6-acetylmorphine (1 mg/mL), 3,6-diacetylmorphine (1 mg/mL) and meperidine (1 mg/mL), purchased from Sigma–Aldrich, Germany. Opiate alkaloids working solutions were prepared from stock solutions in the concentration range of 0.03–3.0 μg/mL. Internal standard working solution was prepared at the final concentration of 3.0 μg/mL.

Calibration samples – spiked blood and brain tissue samples were prepared by adding aliquots of standard working solutions of opiate alkaloids to drug-free (control) blood (0.5 mL) and brain tissue (0.15 g) to final concentrations from 0.03 to 3.0 μg/mL and 0.2 mL of standard working solutions of IS. All calibration samples were extracted according to the method described above.

Quantification was carried out on the basis of the characteristic m/z values of ions for each particular opiate alkaloid. The ratio of the peak areas of opiate alkaloids and that of IS was presented as a function of the substance concentration using linear regression method, the coefficient of correlation being $r^2=0.997–0.999$. The lower detection limit for each of the opiate alkaloids was 0.01 μg/mL, limits of quantitation were 0.03 μg/mL and recovery was in the range of 80–90%.

Statistical analysis

Results are expressed as means ± SD. For testing the difference between groups, Student's t test was used. The difference was considered statistically significant if $p<0.05$. Statistica 7.0 program was used for statistical analysis.

RESULTS

Contents of individual opiate alkaloids: codeine, morphine, acetylcodine, 6-acetylmorphine and 3,6-diacetylmorphine in the samples of brain regions:

Figure 1. Codeine content in brain regions of rat
cortex, brainstem, amygdala and basal ganglia of rats measured 5, 15, 45, and 120 min after the treatment with seized heroin are shown in Figures 1-5.

Figure 2. Morphine content in brain regions of rat

![Morphine Concentration Graph](image)

Figure 3. Acetylcodeine content in brain regions of rat

![Acetylcodeine Concentration Graph](image)

Statistically significant differences with respect to the control were found for the values of concentration of opiates in the blood and brain tissue of rats after 5, 15, 45 and 120 min. Also, statistically significant differences were observed for the values of opiates' concentration in the blood (p<0.05) and brain tissue (p<0.05) after 15 min vs. 5 min, 45 min vs. 15 min, and 120 min vs. 45 min.
The presented results show that the highest content of all opiate alkaloids in all brain regions of rats were measured 45 min after treatment with heroin. The highest concentration values in parts of the brain tissue being measured: for codeine 581.3±120.8 ng/g in amygdala, for morphine 189.2±54.0 ng/g in basal ganglia, for acetylcodeine 245.4±61.0 ng/g in amygdala, for 6-acetylmorphine 319.5±87.0 ng/g in amygdala and for 3,6-diacetylmorphine 20.8±8.2 ng/g in basal ganglia.

After 120 min, a decrease in the opiate alkaloids concentrations is observed in all brain regions of rats. The highest concentration values being measured: for
codeine 143.2±15.0 ng/g in basal ganglia, for morphine 129.4±20.4 ng/g in basal ganglia, for acetylcodeine 22.4±9.5 ng/g in basal ganglia, for 6-acetylmorphine 35.3±14.8 ng/g in cortex and for 3,6-diacetylmorphine 6.2±1.8 ng/g in amygdala.

The overall contents of opiate alkaloids: codeine, morphine, acetylcodeine, 6-acetylmorphine and 3,6-diacetylmorphine in samples of blood and brain regions measured 5, 15, 45, and 120 min after the treatment of rats with seized heroin are shown in Figure 6.

![Figure 6. Total opiate alkaloids' content in blood and brain regions of rat](image)

Figure 6. Total opiate alkaloids' content in blood and brain regions of rat

The presented results show that the highest content of total opiates in the blood samples (2994.5±505.5 ng/mL) was measured 15 min, and in all brain regions of rats 45 min after treatment with heroin, the highest of them being measured in the amygdala (1318.7±121.5 ng/g) and basal ganglia (1025.3±80.7 ng/g). The content of total opiates in the blood after 45 min (1209.7±131.7 ng/mL) is nearly the same as in parts of the brain tissue.

After 120 min, a decrease in the opiates concentrations was observed in rats blood and brain tissue samples, and contents of total opiates were nearly the same (322.9±40.3 ng/mL in blood and 124.8±14.5 – 304.4 ± 42.5 ng/g in brain regions).

The highest values of total opiates in rats brain tissue after 120 min was determined in the basal ganglia (304.4±42.5 ng/g) and cortex (235.8±31.3 ng/g).

**DISCUSSION**

The aim of this work was to examine the regional distribution of opiate alkaloids from seized heroin in the blood and different brain regions of experimental animals at different time periods (5, 15, 45 and 120 min) in order to perform selection of brain regions with the highest content of opiates. Results
could be used in the examination of opiate alkaloids distribution in the human brain tissue, which analysis would contribute to resolving the causes of death due to heroin intake.

To this end, to imitate most closely the use of opiates by intravenous addicts, the experimental animals were treated with seized heroin from the illegal market in Novi Sad.

We developed procedures of preparation and application of the seized heroin to experimental animals and isolation of opiate alkaloids from the biological samples of blood and brain regions (cortex, brainstem, amygdala, and basal ganglia), for which previous investigations (Atweh and Kuhar, 1997a, 1997b, 1997c) showed the highest density of \( \mu \)-opiate receptors.

The highest content of individual (20.8±8.2 - 581.3±120.8 ng/g) and total opiate alkaloids (841.9±53.1 – 1318.7±121.5 ng/g) was determined in all brain regions 45 min after the treatment with seized heroin (the highest concentrations being measured in the amygdala and basal ganglia).

The lowest content of individual (6.2±1.8 – 143.2±15.0 ng/g) and total opiate alkaloids (124.8±14.5 – 304.4 ± 42.5 ng/g) was determined in all brain regions 120 min after treatment with seized heroin (the highest concentrations being measured in the basal ganglia and cortex).

It can be seen from the above that, opiates were most present in the basal ganglia at both measurement times.

Pathways of basal ganglia are divided into two groups, direct and indirect. Direct pathways enable easier flow of information through the thalamus, in other words, they increase the activity of the thalamus, causing excitation of the cerebral cortex. Indirect pathways inhibit the flow of information through the thalamus, they decrease the activity of the thalamus, and consecutively decrease the activity of the cerebral cortex.

Informations pass through the basal ganglia via separated, very complex, paralelly placed neuronal circles. Neuronal circles, that have been studied are: motoric, oculomotoric, posterior – lateral prefrontal, lateral orbitofrontal and limbic. These neuronal circles originate from functionally different parts of the cerebral cortex, pass through certain parts of the basal nuclei, alter the function of different areas in the thalamus, and end in functionally different parts of the cortex.

Mammalian basal ganglia are associated with a variety of functions: motor control, cognition, emotions and learning. Currently popular theories implicate the basal ganglia primarily are relevant action selection, that is, the decision of which of several possible behaviors to execute at a given time (Marieb and Mallat, 2003).

The obtained experimental results are of importance as they offer the possibility of selecting the basal ganglia as a representative sample for the identification and determination of opiates concentration after heroin intake.

The differences in brain tissue disposition might have several explanations, such as variations in fat content or in the density of opiate receptors. Differences in
regional blood flow might also influence the concentration in various brain regions (Strandberg et al., 2004).

The highest content of total opiate alkaloids in blood (2994.5±505.5 ng/mL) was determined 15 min after the treatment with seized heroin.

However, by comparing the opiate concentrations in blood and brain regions after 45 min (1209.7±131.7 ng/mL in the blood, 841.9±53.1 – 1318.7±121.5 ng/g in the brain regions) and 120 min (322.9±40.3 ng/mL in the blood and 124.8±14.5 – 304.4 ± 42.5 ng/g in the brain regions), it can be seen that the values are approximately equal. This indicates that equally with the blood, analysis of brain tissue is of great importance in determining the causes of deaths due to heroin intake.

The obtained results in our study are in agreement with recent investigations indicating the importance of the analysis of brain tissue in the determination of the role of drugs of abuse in the cause of death (Stimpfl and Reichel, 2007).

In conclusion, the observed distribution of opiate alkaloids is of significance in respect of the regional structures of the central nervous system. The results of the present study could be used in the examination of opiate alkaloids' distribution in human biological samples and offer the possibility of selecting the part of brain tissue - basal ganglia, as a reliable sample which analysis would contribute to resolving the causes of death due to heroin intake.

As an initial step in this direction, a database with reliable reference values for drug concentrations in specific regions of the brain should be created.

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REFERENCES

REGIONALNA DISTRIBUCIJA OPIJATNIH ALKALOIDA U MOŽDANOM TKIVU I KRVI EKSPERIMENTALNIH ŽIVOTINJA

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SADRŽAJ

Cilj ovog rada je bila analiza regionalne distribucije opijatnih alkaloida iz zaplenjenog heroina u moždanom tkivu i krvi eksperimentalnih životinja. Dobijeni
rezultati treba da daju doprinos u ispitivanju distribucije opijata u humanim biološkim uzorcima, kako bi se doprinelo razrešenju uzroka smrti prouzrokovane konzumiranjem heroina.

Eksperimentalne životinje (Wistar pacovi) su bile tretirane zaplenjenim heroinom, nakon čega su žrtvovane u različitim vremenskim periodima: 5, 15, 45 i 120 minuta. Sadržaj opijatnih alkaloida (kodeina, morfina, acetilkodeina, 6-acetilmorina i 3,6-diacetilmorina) je određivan u delovima moždanog tkiva (moždanom kori, moždanom stablu, amigdalama i bazalnim jedrima) i krvi životinja metodom gasne hromatografije i masene spektrometrije (GC-MS).

Najveći sadržaj opijatnih alkaloida je utvrđen u krvi nakon 15 minuta, a u delovima moždanog tkiva nakon 45 minuta, od tretiranja životinja heroinom. Maksimalne koncentracije opijata su utvrđene u bazalnim jedrima.

Dobijeni rezultati pružaju mogućnost selekcije ovog dela moždanog tkiva, koji predstavlja reprezentativni uzorak za identifikaciju i utvrđivanje sadržaja opijata.