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ORIGINAL ARTICLE

A study of free and total morphine in heroin death of chronic users

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Summary Heroin is a widely abused substance, involved in a large number of deaths. It has been seen that long-term exposure to street heroin results in an increase in morphine glucuronide metabolites. This suggested us that there might be a relationship between blood morphine metabolites concentrations and heroin-related deaths. In particular, the concentration of morphine glucuronide metabolites could be higher in delayed heroin-related death compared to rapid heroin-related deaths. To evaluate this hypothesis, 53 forensic autopsies of heroin-related death collected between 2016 and 2022 were divided into two groups: chronic user (CU) and non-chronic users (NCU), on the basis of the morphine positivity in hair. The difference of blood free morphine concentrations (FM), blood total morphine concentrations (TM) in blood and bile morphine concentrations (BTM), in the two groups were statistically analysed. Also, the FM/TM ratios were calculated. FM was used to analyse the difference of immediate, subacute and delayed deaths in the two groups. The results showed that the median FM value in the CU group was non-statistically higher than in NCU one. The CU group showed a significantly higher median TM and BTM values, but a significantly lower FM/TM value than in NCU group. Furthermore, the CU group showed a significantly higher percentage of FM in delayed deaths than NCU group. In conclusion, measurements of blood variables indicate a shift in morphine metabolites that could be involved in heroin-related death among chronic users, possibly being a risk factor for delayed causes of death of drug users.

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Introduction

Opioid overdose remains a significant factor contributing to fatalities among heroin users, primarily due to respiratory depression. However, the factors behind heroin-related deaths are still not well understood [1—3]. Concurrent use of other substances and the development of tolerance are likely key contributors to heroin-related mortality [4—7].

Once inside the body, heroin quickly breaks down into 6-monoacetylmorphine (6MAM) and then into morphine. Morphine undergoes conversion into morphine 3-glucuronide (M3G) and morphine 6-glucuoronide (M6G) through UDP glucuronosyltransferase located in the endoplasmic reticulum of hepatocytes. M6G and M3G exhibit distinct differences in their biological activity. M3G is almost entirely inactive, although according some authors its involvement in tolerance phenomena [8], while M6G is considered a potent opioid metabolite [9,10]. There is a growing interest in the role of morphine glucuronide metabolites, particularly M6G, in mediating heroin effects. According to some authors, a considerable proportion of the effects of heroin overdose may be attributed to morphine glucuronide metabolites [11].

While it has been shown that M6G has lesser respiratory depressant effect than morphine [12,13], its accumulation, as seen in cases of renal failure caused by the assumption of large dose of morphine, can lead to increased respiratory depression [14,15]. The onset of action of M6G is delayed compared with the progenitor drug because it crosses the blood-brain barrier more slowly due to its higher polarity [15].

In humans studies, higher concentration of M6G respect to M3G have been observed when comparing plasma concentration of morphine glucuronide metabolites between cases of heroin-related deaths and clinical subjects [16], or between cases of death and living individuals with heroin addiction [17]. Furthermore, it has been noted that long-term exposure to street heroin results in a relative increase in plasma M6G levels compared to M3G [18]. Additionally, repeated injection of heroin in rats lead to an elevated plasma levels of the active metabolite M6G compared to M3G [19,20]. The results of these studies may suggest that metabolite concentrations, particularly M6G, could differ between heroin-related deaths of naïve or occasional users and long-term or chronic heroin users. To our knowledge, there are no studies comparing heroin metabolite concentrations between these two types of heroin-related deaths.

Moreover, according to the literature, the concentration of morphine glucuronide may be higher in heroin-related delayed deaths compared to rapid heroin-related deaths. In delayed deaths, the glucuronidation process may continue for a longer duration respect to rapid deaths. Some studies have suggested the use of the free/total morphine ratio as means of assessing the time of survival, and this ratio was notably higher in rapid death compared to delayed ones [5,21,22]. On the other hand, some studies have associated immediate death with the presence of the morphine metabolite 6-monoacetylmorphine [23,24]. Furthermore, according to data in the literature, the blood concentration of this metabolite in heroin-related deaths could be instable [25,26].

The aim of the present study was to investigate whether long-term heroin use results in quantitative variation of morphine glucuronide metabolites in heroin addict. For this purpose, selected cases of heroin-related deaths were classified into two groups: long-term or chronic users (CU) and no longer or infrequent users (NCU), based on the results of hair analysis [27] and bile morphine concentration [28]. The concentration of free morphine, total morphine,free/total ratio and total morphine in bile were measured in both groups.

A second aim of the present work was to differentiate between rapid to delayed opiate-related deaths in the two groups. For this purpose we adopted the classification proposed by Al-Asmary and Andersen. These authors distinguish immediate, subacute (occurring in less than 3 hours), and delayed heroin-related deaths (occurring more than 3 hours after assumption) based on blood free morphine concentration levels (immediate > 0.5 μg/mL; subacute < 0.5 and > 0.2 μg/mL; delayed < 0.2 μg/mL) [29].

Materials and method

Case material

The cases used in this study were heroin overdoses categorized as ''certainly fatal''. These cases were examined at the Toxicological Laboratory of Macerata in the period from January 2016 to December 2022. The diagnosis in all cases was established through a combination of autopsy findings, toxicological testing of blood, bile and hair samples, microscopic examinations and consideration of the circumstances surrounding the deaths (including knowledge of individuals as heroin users and indications of recent heroin use, including analysis of several drugs and paraphernalia).

Polydrug assumption, typical of heroin users, can increase the inherent toxicity of opioids [5,6]. For this reason, we excluded cases where substances other than morphine were detected in blood samples. Cases involving putrefaction or death during clinical treatment were also excluded from the study.

The cases of heroin-related deaths ($n = 56$) were divided into two groups: long-term or chronic user (CU, $n = 37$) and former or occasional user groups (NCU, $n = 19$). To distinguish between the two groups, we relied on the presence or absence of morphine and its metabolites in hair matrix. Hair analysis has been used widely for the detection of drug of abuse, providing evidence of prolonged drug exposure and offering a means to discriminate between chronic and non-chronic drug use [27].

Chemicals and reagent

Nalorphine (internal standards for opiates) and sulfosalicylic acid were purchased from Sigma along with N-Methyl, N-trimethylsilyl-trifluoroacetamide (MSTFA). Morphine was purchased from LCG standards. Methanol, dichloromethane, prop-2-ol, ammonium hydroxide, all of which were of reagent grade, were purchased by Carlo Erba. Isolute HCX cartridges (10 mL capacity, 130 and 300 mg) were obtained from International Sorbent Technology.

Toxicological analysis of blood and bile

In all cases of fatal intoxication, toxicological analyses were conducted on femoral blood and bile. As first step of analysis, cadaveric blood was deproteinated using sulfosalicylic acid. The blood sample was vortexed to allow the optimal contact between the blood and the acid. Subsequently, the sample was centrifuged at 4000 rpm for 10 min. The supernatant was collected and divided into two equal portions, both of which were spiked with internal standard nalorphine.

One aliquot was immediately assayed without hydrolysis to determine free morphine concentration (FM) in blood. The second portion of deproteinized blood, and if available, bile from each individual, underwent acid hydrolysis with 1 mL of 0.1 M hydrochloric acid, incubated overnight at 45 °C for quantify total morphine concentrations (TM and BTM). Subsequently, the samples were neutralized with 2 N sodium hydroxide and buffered with 2 mL of Tris(hydroxymethyl)aminomethane buffer at pH 8.1. Then, the sample was then centrifuged at 4000 rpm for 5 min.

Isolute HCX 300 mg were used for solid phase extraction (SPE). The columns were conditioned with 2 mL of methanol followed by 2 mL of water. The samples were slowly drawn through the columns under low vacuum. The columns were washed sequentially with 2 mL of water, 3 mL of 0.1 N HCl, and 2 mL of methanol, and then dried for 5 min under full vacuum. The analytes were eluted with 2 mL of a dichloromethane/isopropyl alcohol solution (8:2) with 2% ammonium hydroxide, freshly made daily. The eluate was transferred to 4 mL vials and evaporated to dryness.

The residue was reconstructed with 50 μL of N-methyl-N-trimethyl-silyl-trifluoroacetamide (MSTFA), incubated for 15 min at 75 °C in sealed vials and ready for injection in gas chromatograph-mass spectrometry (GC-MS).

Toxicological analysis of hair

The hair sample collected during the autopsy, was cut to from the posterior vertex area to the scalpand stored in a plastic bag at—20 °C until analyzed. The three most proximal centimeters of the hair was used to analyze the opiate use over the 3 months preceding death [2]. The hair was washed once with distilled water and twice with methanol and cut into small pieces, less than 1 mm in length. Fifty mg of sample were weighed in conical tubes and 1 mL of 0.1 M hydrochloric acid and internal standard nalorphine were adeed for extraction. The sample was neutralized, buffered at pH 8.1, and prepared for SPE with isolute HCX 130 mg cartridge. The extraction procedure followed was the same used for blood and bile.

GC-MS analysis

All the analyses were performed using the Thermo GC-MS Polaris-Q. For each reconstructed sample, 1 μL was injected into the system with the following operating conditions: capillary column (ZB 5 MS 30 m × 0.25 mm × 0.25 μm); carrier gas was helium at flow rates of 1.5 mL/min. Injector temperature was 200 °C. The transfer line and the source temperature were 250 and 230 °C respectively. The oven temperature profile was programmed to increase from 100 to 180 °C at 40 °C/min, and then ramped at 10 °C/min to a final temperature of 310 °C, which was held for 5 min. The injection mode was splitless. EI (70 eV) was used for ionization. Mass spectra were recorded in the range m/z 70—500. The ions chosen were m/z 429, 414, 401, 324 for morphine-TMS and 455, 414, 440, 324 for nalorphine-TMS. The quantitative determinations of the samples were performed in accordance with the validated procedures [28,30].

Statistical analysis

The medians of the following variables were compared between the groups: FM and TM morphine in blood, and biliary total morphine (BTM) and the FM/TM ratios. Differences in the distribution of data between the two groups were evaluated using the Mann-Whitney two-tailed U test. The differences in the percentage of FM between the groups were evaluated using χ^2 test.

Results

The results of statistical analysis of the distribution of FM, TM, FM/TM ratio and BTM are presented in Table 1.

The median FM value was observed to be higher in the NCU group compared to the CU group, although this difference did not reach statistical significance. In contrast, the TM median value was significantly lower in the CU group than in the NCU group, indicating that heroin metabolites are reduced in this latter group. This difference is further evident when comparing the FM/TM ratio between the two groups. This ratio was found to be significant lower in the CU groups than that in the NCU group.

Regarding BTM, the CU group showed a significant higher median value than the NCU group. The application of the Al-Asmari classification to distinguish and analyse the timespam between heroin use and death is shown in Table 2.

The results showed that the percentage of delayed heroin-related death among CU was significantly higher compared to delayed heroin-relayed death of NCU ($\chi^2 = 36.12$, $P < 0.005$). Conversely, the CU groups exhibited a significantly lower percentage of immediate and subacute heroin deaths in comparison to the NCU groups ($\chi^2 = 16.04$, $P < 0.05$; $\chi^2 = 9.90$, $P < 0.05$). No significant difference was observed between the immediate and subacute heroin deaths in the two groups ($\chi^2 = 0.67$, NS).

Discussion

The selection of subjects who died from a heroin overdose in this study was based on whether they had a history of repeated heroin use or were naïve to heroin use, determined by analysing the presence of morphine in hair samples. Additionally, in cases where morphine was present in hair samples, reports from the police or public prosecutors were considered. This selection criterion was supported by the results related to bile, where the CU group showed higher levels of bile morphine compared to the NCU group. Previous studies have evidenced higher bile morphine concentrations

Table 1 Medians, minimum and maximum for selected variables in the chronic heroin users (CU), non-chronic heroin users' groups and *P* values for the Mann-Whitney U Test.

Group		FM (μg/mL)	TM (μg/mL)	FM/TM (μg/mL)	BTM (μg/mL)
CU	Median	0.54	0.89	0.5	24.5
	Minimum	0.20	0.13	0.12	0.6
	Maximum	1.28 ($n = 35$)	2.21 ($n = 35$)	0.98 ($n = 35$)	46.5 ($n = 30$)
NCU	Median	0.45	0.43	0.85	12.9
	Minimum	0.14	0.37	0.16	0.8
	Maximum	1.54 ($n = 18$)	1.02 ($n = 18$)	0.91 ($n = 18$)	29.3 ($n = 16$)

FM: free morphine; TM: total morphine; BTM: total morphine in bile.

Table 2 FM distribution in the chronic heroin users (CU), non-chronic heroin users' groups and *P* values for χ^2 test.

Group	FM > 0.5 (μg/mL)	FM < 0.5 (μg/mL)	FM < 0.2 (μg/mL)
CU (%)	14.28	22.86	62.86
NCU (%)	38.89	44.44	16.67
	$P < 0.05$	$P < 0.05$	$P < 0.05$

FM: free morphine.

in established heroin addicts, while newcomers to heroin use or those returning after a period of withdrawal displayed low values [23]. This suggests an association between high levels of morphine in bile and chronic opiate use. The results of this study indicate that median FM concentration of heroin long-term users is higher than that in naïve or no longer users, although this difference did not reach statistical significance. However, the long-term users group showed a significantly lower value of blood TM compared to the naïve group, indicating an increase in the morphine glucuronide metabolite level. This is supported by the FM/TM ratio results. The CU group showed a significantly lower value of FM/TM ratio than that of the NCU group.

According to the literature, the FM/TM ratio in blood can be used to predict a fatal heroin intake [31,32] or to estimate the time elapsed between the assumption and death [21,23]. Higher FM/TM ratios could be associated with rapid death because less time is available for the transformation of morphine into its metabolites. Furthermore, the ratios between the parent drug and metabolite are influenced to a lesser extent by the dose of heroin [33], and the individual pharmacokinetic of morphine and its glucuronides play a role in determining the FM/TM ratio after heroin intake [31]. Additionally, it has been shown in literature lower free morphine concentration in delayed heroin death compared to rapid heroin death [24,34]. The Al-Asmary classification correlates the blood free concentration of heroin death with the time-spam between administration of heroin and death. Application of the free morphine of the CU and NCU groups to this classification indicates a higher percentage of the CU group falling in the delayed death respect the NCU, which showed major percentage in immediate or subacute death. Chronic exposure to street heroin has been shown to result in a relative increase in the concentration of active metabolites M6G [18,35], with no effect [35] or a decrease of M3G concentration [18]. M6G and M3G exhibit significant differences in their biological activity, with M3G being almost completely inactive, although some authors suggest its involvement in the development of tolerance phenomena [8,9,35]. On the other hand, M6G is considered a potent opioid metabolite [9,10]. There is an increasing interest in the role of morphine glucuronide metabolites, especially M6G in mediating heroin effects. Opioid-dependent individuals have been shown to have higher concentration of M6G compared to non-opioid-dependent subjects [18,36]. According to the literature, a considerable proportion of the effects in heroin overdose may be attributed to morphine glucuronide metabolites [11]. These metabolites may have a reduced respiratory depressant effect of the hypoxic ventilator response than morphine [12,13,15], but they exhibit a similar reduction in the ventilator response to carbon dioxide. Respiratory depression resulting from heroin intoxication is due to the suppression of brainstem respiratory centres, primarily through a decrease in the ventilator response to carbon oxide. It is also worth noting that high levels of plasmatic M6G might lead to respiratory depression [14], with a different time course respect to that induced by morphine due to the slow transfer of M6G between the plasma and the effect compartment [15].

Our data, comparing heroin-related death of chronic and not chronic users, provide support for the hypothesis that morphine metabolites, especially M6G, could be an important factor involved in delayed heroin deaths among chronic users [34,36].

Conclusion

In conclusion, the results obtained in this study, though limited by the number of cases available and uncontrolled variables (such as doses, survival times, etc.), suggest that chronic heroin use may enhance morphine metabolism.

Further studies may clarify whether this enhancement is related to an increase in the concentrations of the active metabolite M6G in delayed heroin-related deaths among chronic/long-term heroin users, as the slower onset of action of M6G could be considered a risk factor for delayed heroin-related deaths in this population.

Disclosure of interest

The authors declare that they have no competing interest.

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