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Original Article / Оригинални рад

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The importance of 6-MAM levels and morphine/codeine ratio in diagnosis of death among drug addicts

Значај концентрације 6-MAM и односа морфин/кодеин у дијагностици смрти наркомана

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SUMMARY

Introduction/Objective Heroin is metabolized to 6-monoacetylmorphine (6-MAM) and morphine. The aim of this study is to examine 6-MAM, morphine and codeine relationships in order to distinguish deaths related to heroin consumption from deaths related to morphine and/ or codeine consumption.

Methods The autopsy blood and urine samples from 45 opioid drug addicts were examined. Gas chromatography/ mass spectrometry was applied to evaluate morphine, 6-MAM and codeine. Two groups were formed: 6-MAM positive (n = 35) and 6-MAM negative (n = 10).

Results Compared to 6-MAM negative group, blood morphine levels were higher in 6-MAM positive group (p = 0.022), while blood codeine levels were similar (p = 0.575). In 6-MAM negative group the blood morphine/codeine ratio was 8.3 and in 6-MAM positive group it was 4.3. There was no difference between groups regarding urine morphine levels (p = 0.859). The urine morphine/codeine ratio was 6.2 in 6-MAM negative group, whilst 32.2 in 6-MAM positive group. In the blood samples, morphine and codeine concentrations were significantly correlated (r = 0.607; p = 0.006). In urine samples, correlations between morphine and codeine (r = 0.766; p < 0.001), morphine and 6-MAM (r = 0.650; p < 0.001), as well as codeine and 6-MAM (r = 0.620; p < 0.001), were also significant.

Conclusion Analysis of 6-MAM and morphine/codeine ratio in blood and urine autopsy samples may be used as diagnostic tools to distinguish deaths related to the consumption of different opioid drugs.

Keywords: autopsy; heroin; 6-MAM; morphine; codeine

САЖЕТАК

Увод/Циљ Хероин се у организму метаболише у 6-моноацетилморфин (6-MAM) и морфин. Циљ рада је да испитујући концентрације 6 MAM, морфина, кодеина, и њихових односа диференцијално дијагностички дефинишемо разлику између смрти које су наступиле у вези са уношењем хероина у организам, од оних које су наступиле у вези са коришћењем морфина и или/кодеина.

Методе Код 45 обдукованих наркомана код којих је у узорцима крви и урина однос вредности концентрација морфин/кодеин >1, гасном хроматографијом/масеном спектрометријом одређиване су концентрације морфина, 6-MAM-а и кодеина. Формиране су две групе испитаника: 6-MAM позитивна (*n*=35) и 6-MAM негативна група (*n*=10).

Резултати У поређењу са 6-MAM негативном групом, концентрација морфина у крви је била значајно већа у 6-MAM негативној групи (*p* = 0.022), док се вредности кодеина у крви нису разликовале (*p* = 0.575). У 6-MAM негативној групи однос морфин/ кодеин у крви је био 8.3, док је у 6-MAM позитивној био 4.3. Није било значајне разлике у концентрацији морфина у урину међу групама (*p* = 0.859). Однос морфин/кодеин у урину у 6-MAM негативној групи је био 6.2, док је у 6-MAM позитивној групи био 32.2. Концентрације морфина и кодеина у крви су значајно корелирале (*r* = 0.607; *p* = 0.006), као и концентрације морфина и кодеина у урину (*r* = 0.766; *p*<0,001), морфина и 6-MAM у урину (*r* = 0.650; *p*<0.001) и кодеина и 6-MAM у урину (*r* = 0.620; *p*<0.001).

Закључак Присуство 6-MAM у узорцима телесних течности и однос морфин/кодеин могу бити корисни индикатори и послужити у дијагностици смрти у вези са узимањем опијата.

Кључне речи: обдукција; хероин; 6-MAM; морфин; кодеин

INTRODUCTION

Heroin (3,6-diacetylmorphine) is an opioid drug synthesized by acetylation of morphine derived from crude opium. Beside morphine, the crude opium contains several other alkaloids, including codeine (3-methoxymorphine). During morphine synthesis, codeine also undergoes

acetylation reaction yielding 6-acetylcodeine. As the result, heroin often contains a certain quantity of codeine as an impurity.

Heroin and codeine are actually pro-drugs, which are both metabolized to the common active form – morphine. Heroin is metabolized to morphine via several intermediary products. After intravenous injection heroin is for maximum 3 minutes converted to 6-monoacetylmorphine (6-MAM) by hydrolysis of one acetyl group. Thereafter, 6-MAM is metabolized to morphine during 20 minutes [1] to 3 hours [2]. Codeine is converted to morphine by o-demethylation via hepatic cytochrome P450-containing enzyme – CYP2D6 [3]. Compared to heroin metabolism to morphine, the conversion of codeine to morphine is a relatively slow process, and in codeine intoxication it may take 24 hours before measurable morphine levels appear in the blood [4].

Positive detection and measurement of heroin metabolites in autopsy body fluids, without previous knowledge of death circumstances, can be ascribed to heroin consumption, but also to morphine intoxication which may occur during medical treatment of acute and chronic severe pain. According to the literature data there are two indicators of heroin exposure in living individuals: the presence of 6-MAM in body fluids and tissues, and morphine/codeine ratio > 1. The latter is considered to be especially important in cases where 6-MAM was not detected, as well as in cases suspected for codeine intoxication [5, 6, 7]. However, these relationships seem to be by far complex in forensic autopsy samples, and with few exceptions, in Serbian drug addicts poorly investigated.

This study evaluated blood and urine toxicological indicators of heroin usage, 6-MAM and morphine/codeine ratio, obtained during medicolegal death investigation of cases suspected for heroin or morphine intoxication, in order to distinguish deaths related to heroin consumption from those related to the consumption of morphine and/ or codeine.

METHODS

This study was performed at the Institute for Forensic Medicine "Milovan Milovanovic", School of Medicine, University of Belgrade, after ethical institutional clearance was obtained. From January 2006 to December 2015 a total of 12817 autopsies were done, of which 351 autopsy cases of adult drug addicts, both sexes. The inclusion criteria in the study were: (1) the death suspected for accidental heroin intoxication, and (2) in addition to morphine, the presence of codeine and/ or 6-MAM in autopsy blood and urine samples. Forensic autopsy files, heteroanamnestic data from closest relatives, police records, and medical history files, for all studied cases were also collected. Of all autopsies performed in that period, 219 were associated with psychoactive substances abuse, but only 134 cases fulfilled the inclusion criteria.

The postmortem blood and urine samples were taken with sterile syringe into chemically clean glass tubes. Approximately 50 – 100 *mL* of blood was taken from the femoral vein, and about 50 – 100 *mL* of urine was taken through the intact urine bladder wall. Samples were thereafter aliquoted and kept in closed tubes at $-20^0\,C$, for no longer than 4 weeks before analyzing.

Morphine, codeine and 6-MAM were screened and quantitatively analyzed by gas chromatography (GS) with mass spectrometry (MS) detection, using a headspace technics, adopted for opiates [8]. We used Agilent 7000 GC/MS triple quadrupole gas chromatograph (Agilent Technologies, Inc. Santa Clara CA, USA), connected to the flame-ionization MS detector. Opiates were extracted from specimens according to instructions [8], using a capillary extraction column Oasis MCX-3cc (Waters Oasis®, MA, USA). Free morphine and codeine were screened in the range 40-500, and a specific m/z ratio was used for the detection of a target ion. All positive specimens were further analyzed according to manufacturer's instructions [9], using the selective ion monitoring mode. Morphine was determined at m/z 429 (retention time 11.60 min), codeine at m/z 371 (retention time 11.19 min), and 6-MAM at m/z 399 (retention time 12.47 min). Concentrations of morphine, codeine and 6-MAM were calculated from correspondent standard curves.

The data were processed using the statistical program package SPSS, version 22 (SPSS Inc. Chicago, IL, USA). Data were presented according to the standard descriptive statistics. Discontinual variables were expressed as frequencies and continual variables as median values (and range). Differences between groups were tested by Mann-Whitney U-test. Correlation analysis was accomplished by calculation of Spearman's coefficient. Statistically significant finding was considered if $p < 0.05$.

RESULTS

By screening of autopsy samples from 219 death cases associated with the use of psychoactive substances, the detection of morphine alone occurred in 134 cases (63.0%), and in 63 other cases (28.8%) morphine was combined with another psychoactive substances, most frequently with codeine (56 cases; 25.6%). The results are presented in Table 1.

Samples where morphine alone was detected (n=134) were further quantitatively analyzed. Beside morphine, detectable quantities of codeine and/ or 6-MAM were found in 49 cases. The morphine/codeine concentration ratio (M/C) was calculated. In 45 cases the M/C ratio in the blood and/ or urine was > 1.0, and 4 urine samples had M/C ratio < 1.0. Of 45 cases with M/C ratio > 1.0, measurable levels of 6-MAM in body fluids were found in 35 cases (77.8%), and those comprised the

6-MAM positive group. In other cases (n=10) no measurable 6-MAM was detected, and these comprised the 6-MAM negative group. The results are presented in Table 2 and Table 3.

Median values of blood morphine and codeine, and urine morphine and codeine concentrations in 6-MAM positive and 6-MAM negative groups are presented in Table 4. As presented, blood morphine levels significantly differed between groups and were approximately two times higher in 6-MAM negative than in 6-MAM positive group (U=3.5; p =0.022). Concentrations of blood codeine were similar in both groups (U=19.0; p = 0.575). However, in 6-MAM negative group the median blood M/C ratio was 8.25 and in 6-MAM positive group the median blood M/C ratio was 4.25.

Compared to 6-MAM negative group, median values of urine morphine levels were somewhat higher in 6-MAM positive group (Table 4), but the difference between groups was not significant (U = 134.0; p = 0.859). Compared to 6-MAM negative group, the medium urine codeine concentrations were about three times lower in 6-MAM positive group. The difference between groups regarding urine codeine levels were also not significant (U = 129.0; p = 0.734). However, in 6-MAM negative group the median urine M/C ratio was 6.2, whilst in the 6-MAM positive group the median urine M/C ratio was 32.2, and this difference was statistically significant ($p < 0.001$).

We further analyzed relationships between morphine, codeine and 6-MAM within each body fluid compartment. The results are presented in Table 5. In the current study there were significant correlations between blood morphine and blood codeine levels (r = 0.607; p = 0.006), urine morphine and urine codeine levels (r = 0.766; $p < 0.001$), urine morphine and urine 6-MAM levels (r = 0.650; $p < 0.001$), and between urine codeine and urine 6-MAM levels (r = 0.620; $p < 0.001$).

DISCUSSION

The M/C ratio is now considered to be a useful parameter to distinguish the heroin associated death from the death associated with codeine abuse, since in the latter cases the M/C ratio is often ≤ 1.0 [7, 10]. Beside morphine, the presence of codeine and/or 6-MAM was detected in 49 of 134 cases (36.5%) in the current study. In 45 cases the M/C ratio in the blood and/ or urine was > 1.0, and in 4 cases the urine M/C ratio was < 1.0. Of these 45 morphine and codeine positive cases, in 35 cases (77.8%) we also detected measurable amounts of 6-MAM in the blood and/ or urine samples. The M/C ratio in morphine and codeine positive cases varied from 2.5 to 13.0. Previously, the M/C ratio was determined exclusively in the blood samples [5, 11, 12], and more recently the M/C ratio was calculated from both blood and urine samples [4].

For identification and measurement of 6-MAM, as currently most accurate molecular fingerprint of heroin exposure, it is often recommended to employ urine samples. The main reason is that, in contrast to the blood, 6-MAM once excreted into the urine is not further metabolized in organism [13, 14]. In Norway, Konstantinova et al have been investigating the M/C ratio in a large number of cases (N=2438), positive for 6-MAM and/or morphine and codeine in the blood and urine samples [4]. In that study in 98% of blood samples and 96% of urine samples the M/C ratio was > 1.0, and varied from 5 to 10, which is largely in accordance to our findings. Moreover, one Swedish study of 747 cases related to heroin abuse also reported that in 98.9% 6-MAM positive cases the M/C ratio was > 1.0, although the M/C ratio was actually very high, with median value found to be 11.0 [6]. Estimating the survival times in acute heroin intoxication, Drake and Duflou have reported 6-MAM in 43% cases [1]. That study also reported that 6-MAM positive cases have two times higher morphine levels than 6-MAM negative cases, which was also found in the current report (Table 4). Ceder and Jones have been evaluated 6-MAM, morphine, codeine and M/C ratio in living persons imprisoned for driving under the influence of drugs [5]. Of 675 blood samples in that study, 6-MAM was present in 16 (2.3%), and of 339 urine samples 6-MAM was recorded in 212 samples (62%). When 6-MAM was detected in urine, the blood M/C ratio was always > 1.0, and varied from 1 – 66, with median value of 6.0 [5].

However, the M/C ratio should be analyzed through the prism of free morphine and codeine levels. In the current study we observed high median levels of blood morphine in both 6-MAM positive (0.17 *mg/L*) and 6-MAM negative cases (0.33 *mg/L*), and even higher median levels of urine morphine in 6-MAM positive (3.33 *mg/L*) and 6-MAM negative cases (2.37 *mg/L*), respectively. In contrast to that, median values of either blood or urine codeine levels were much lower and were not related to the 6-MAM status (0.04 *mg/L*, and 0.11 mg/L, respectively). These results are in accordance with several other studies who reported that blood free morphine levels vary from 0.22 to 0.60 *mg/L* and blood free codeine levels vary from 0.01 to 0.07 *mg/L* after heroin exposure [4, 6, 7, 8].

We observed that compared to the 6-MAM negative group, concentration of the blood free morphine was lower in the 6-MAM positive group (0.33 *mg/L* vs. 0.17 *mg/L*). Although this was opposite from several other studies who reported higher blood morphine levels in 6-MAM positive group [4], our findings are in accordance with Ceder and Jones who also found higher median values of blood morphine in 6-MAM negative group [5].

Also, morphine levels were higher than codeine levels in all 6-MAM positive cases and all 6-MAM negative cases with M/C ratio > 1, either in the blood or urine samples (Table 4). Moreover, bloo morphine and blood codeine levels, as well as urine morphine and urine codeine levels, were significantly correlated (Table 5). These findings are somewhat expected, and may be partly explained by the fact that illegal production of heroine from crude opium is necessarily accompanied by

increased presence of codeine, as an impurity, thus heroin drug addicts consume some codeine as well. In the 6-MAM positive group there were statistically significant correlations between 6-MAM and morphine, as well as between 6-MAM and codeine in urine samples. Similar results were reported on cadaveric samples as well as on samples from living drug addicts [4, 5, 7], thereby confirming that morphine and codeine levels in the body fluids are tightly related to the quantity of heroin taken with the dose.

In four 6-MAM negative cases the blood M/C ratio was > 1.0, whilst the urine M/C ratio was < 1.0, with median of 0.55. Konstantinova et al also reported low values of M/C ratio in 2% of blood and 4% of urine samples but among 6-MAM positive cases [4], while Ceder and Jones reported M/C ratio < 1.0 in 15% cases of 6-MAM positive living, imprisoned drug addicts [7]. One probable explanation of such divergent M/C ratio findings observed among 6-MAM negative cases could be related to longer survival time, during which the blood 6-MAM could undergo further metabolic reactions yielding products that were not targeted in our study.

Codeine is an opioid analgesic drug. However, median concentration of free codeine in 4 cases with urine M/C ratio < 1.0 in our study was 0.67 mg/M (0.31 – 1.10 *mg/L*). This was 2.4 times higher than free morphine levels in the same group (median 0.28 *mg/L*: range: 0.024 – 0.94 *mg/L*), and several times higher than therapeutic codeine dose. Pharmacokinetic studies have well documented that the blood concentration of free codeine is several times higher than free morphine after one or more codeine doses [9, 15, 16]. Konstrad et al have reported, for example, that ingestion of 100 mg codeine phosphate gave rise to the free blood codeine to 0.183 *mg/L* [17], while in the study of Quiding et all ingestion of 60 *mg* codeine phosphate gave rise to blood codeine of 0.115 *mg/L* [18]. Ceder and Jones reported blood free codeine levels of 0.180 mg/L in living drug addicts with M/C ratio < 1.0 [5]. These and our results point that a low M/C ratio probably indicate that codeine was deliberately used some time before the death, or, more likely, that codeine as an impurity was highly present in the heroin dose.

CONCLUSION

The current study of the death related to chronic heroin exposure indicates that the presence of 6-MAM and M/C ratio in body fluids may be used as reliable tools to differentiate cases in which is unclear whether the death resulted from (chronic) heroin and morphine consumption for non-medical (recreational) reasons, or the death resulted from accidental therapeutic usage of morphine.

NOTE

Manuscript is a part of a doctoral thesis

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Table 1. Opiates and their combinations in drug addicts died from abuse of psychoactive substances from January 2006 to December 2015

Psychoactive substance(s)	Number of cases (n)	Percent (%)
Morphine	134	63.0
Codeine	2	0.9
Methadone	4	1.8
Others	1	0.4
Morphine + codeine	56	25.6
Morphine + methadone	2	0.9
Morphine + tramadol	2	0.9
Morphine + methadone + tramadol	1	0.5
Morphine + codeine + tramadol	1	0.5
Morphine + codeine + methadone	1	0.5
Unknown	11	5.0
Total	219	100.0

Table 2. Distribution of opioid addict cases according to the presence of 6-MAM

Presence of 6-MAM	**Number of cases (n)**	**Percent (%)**
6-MAM negative	10	22.2
6-MAM positive	35	77.8
Total	45	100.0

Table 3. Distribution of opioid addict cases with M/C ratio > 1, according to the presence of 6-MAM in the blood and urine

Presence of 6-MAM	**Body fluid**			
	Blood		**Urine**	
	n	**%**	**n**	**%**
6-MAM negative	3	15.8	9	22.5
6-MAM positive	16	84.2	31	77.5
Total	19	100.0	40	100.0

Table 4. Morphine and codeine concentrations in postmortem body fluids according to the presence of 6-MAM

Opioids in body fluids (μg/ml)	6-MAM n body fluids			
	6-MAM negative		6-MAM pozitivna	
	n	Median (range)	n	Median (range)
Morphine (blood)	3	0.33 (0.23–0.39)	16	0.17 (0.03–0.41)
Morphine (urine)	9	2.37 (0.03–17.20)	31	3.33 (0.10–44.28)
Codeine (blood)	3	0.04 (0.02–0.11)	16	0.04 (0.004–0.090)
Codeine (urine)	9	0.38 (0.01–4.93)	32	0.11 (0.01–8.65)

Table 5. Correlation analysis for **relationships between morphine, codeine, and 6-MAM within each body fluid compartment**

Drug	Body fluid		Codeine (*μg/mL*)		6-MAM (*μg/mL*)	
			Blood	**Urine**	**Blood**	**Urine**
Morphine (*μg/mL*)	Blood	r	0.607		-0.162	
		p	0.006*		0.580	
		n	19		14	
	Urine	r		0.766		0.650
		p		<0.001*		<0.001*
		n		40		29
Codeine (*μg/mL*)	Blood	r			0.114	
		p			0.699	
		n			14	
	Urine	r				0.620
		p				<0.001*
		n				28

Spearman's correlation coefficient (r) was calculated, and significant relationships were marked (*)

