Journal of Chromatography, 422 (1987) 267–273
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3852

Note

Concentrations of free and conjugated morphine in blood in twenty cases of heroin-related deaths

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(First received April 6th, 1987; revised manuscript received June 25th, 1987)

The steadily increasing abuse of drugs has led to a great number of heroin-related deaths. The interpretation of toxicological analysis in these cases is always required by the legal authority to conclude whether the death is directly due to an intravenous injection of heroin.

Heroin is rapidly deacetylated after injection to form monoacetylmorphine, which is further converted to morphine [1]. Thus, it is generally agreed that toxicological analysis of blood in heroin-related deaths should concentrate on the search for morphine [2]. The major part of a given dose of heroin in a human being exists as free morphine and conjugated morphine (mainly 3-morphine glucuronide) [3,4]. Free morphine has an elimination half-life of 2–4 h [5], whereas conjugated morphine, formed in the liver from free morphine, has an enterohepatic recirculation which accounts for nanogram levels of morphine in blood for more than 24 h [6–8].

The concentration of morphine in blood varies considerably owing to variations in the amount of heroin injected and in the time elapsed after the injection [3,6,9,10]. Also, it is extremely difficult to establish the significance of the concentration of morphine found in blood, because the toxic or lethal action of the drug is highly dependent upon the degree of tolerance which the individual may have developed [11,12].

The available information on toxic and lethal levels of morphine in blood in heroin fatalities is diverse and sometimes confusing. The confusion arises from the different techniques used for the analysis, which makes the comparison of data very difficult [13]. The analytical extraction of whole blood with organic solvents will yield only the free morphine fraction [14–17], whereas the same

0378-4347/87/$03.50

extraction after hydrolysis will yield both free and conjugated morphine [11,12]. The recovery of morphine from the conjugated form depends on the hydrolysis procedure [18,19]. Techniques such as radioimmunoassay (RIA), which provides the detection and quantification of both free and conjugated morphine in a single step [20], enable the analyst to avoid the hydrolysis step, but these techniques lack specificity and the quantitative results do not correlate with those obtained by other techniques [21].

On the other hand, literature concerning morphine concentrations in plasma in heroin-related deaths is scarce, probably due to the difficulty of obtaining plasma from partially hemolysed blood of cadavers.

In this paper we present the concentrations in blood of free and conjugated morphine as well as total morphine levels in plasma in twenty cases of heroin-related deaths. We attempt to explain some of the previously published results and we try to present data which could be of aid in the forensic interpretation of death.

EXPERIMENTAL

Samples

From the bodies of twenty persons, whose death was suspected to be heroin-related, blood samples were obtained during routine autopsies by various pathologists over a six-month period. Information was provided by the police report and the judicial inquest. Each blood sample was refrigerated as soon as possible after collection. Samples in which the hemolysis process was advanced were excluded. One part of the blood was transferred into a conical tube and was centrifuged at 1600 *g* for 5 min. The upper plasma layer was then transferred to a clean glass tube and refrigerated until used. The amount of plasma obtained never exceeded 3 ml.

Drugs and chemicals

Morphine hydrochloride and nalorphine hydrochloride were obtained from the National Pharmaceutical Service of Drugs of Spain, which is supplied by the World Health Organisation Centre for chemical reference substances (Geneve, Switzerland). β-Glucuronidase from *Helix pomatia* (100 000 Fishman units per ml) was bought from Boehringer Mannheim (Mannheim, F.R.G.). Sep-Pak C_{18} cartridges were purchased from Waters Assoc. (Milford, MA, U.S.A.). Columns packed with Extrelut silica were purchased from Merck (Darmstadt, F.R.G.). Heptafluorobutyric anhydride was supplied by Fluka (Buchs, Switzerland). All other chemicals and solvents were of the purest grade available (Merck, Scharlau, F.R.G.).

Enzyme immunoassay for plasma

Enzyme immunoassay analyses were performed on an enzyme multiplied immunoassay technique (EMIT) Autolab 5000 (Syva, Palo Alto, CA, U.S.A.) using original reagents (opiate assay). Plasma samples were used after separation from the blood by centrifuging, either directly or prepared as follows. Plasma (1 ml)

was transferred into a conical tube, and 2 ml of acetonitrile were added. After mixing and centrifuging, the supernatant was transferred to a clean glass tube with tapered bottom and evaporated to dryness under a stream of nitrogen at 50°C. The residue was reconstituted with 0.5 ml of the buffer supplied with the EMIT reagents (0.055 *M* Tris buffer, pH 8). Both the plasma and reconstituted extract of plasma were diluted with the same buffer as needed to bring the drug concentration into the range of the EMIT calibrators.

Hydrolysis and extraction procedure for total morphine

For the hydrolysis we followed the procedure suggested by Curry [22] and studied by Predmore et al. [19]: 0.5 ml of whole blood or plasma were added to 5 ml of acetate buffer (pH 5.5) and 0.05 ml β-glucuronidase in a 15-ml screw-cap glass tube. The sample was vortex-mixed and the tube was incubated at 37°C for 40 h. After cooling, 0.05 ml of 3.5 *M* sodium hydroxide and 4 ml of borate buffer (pH 9) were added along with 0.05 ml of a nalorphine solution (0.54 mg per 100 ml) which was used as internal standard (I.S.). The sample was then extracted in a C_{18} Sep-Pak cartridge, carefully following the procedure described by Drost et al. [23]. We purposely avoided the use of enzyme sulfatase, because we were only interested in the cleavage of morphine glucuronide, which is the main metabolite of morphine in blood.

Extraction procedure for free morphine

We followed the procedure described by Edlund [24] with slight modifications: to 10 ml of whole blood, 0.05 ml of a nalorphine solution (10.8 mg per 100 ml) were added as internal standard. The pH was then adjusted to 9 with borate buffer. The sample was vortex-mixed for 10 min and poured into an extraction column packed with Extrelut silica. After 10 min, elution was carried out with 20 ml of a 10% solution of isopropyl alcohol in chloroform. The solvent was evaporated under a stream of nitrogen at 50°C.

Gas chromatographic (GC) analysis of morphine

For the GC analysis of morphine we prepared heptafluorobutyryl derivatives: 0.01 ml of heptafluorobutyric anhydride (HFBA) was added to the extraction residue of blood, hydrolyzed blood or hydrolyzed plasma, along with 0.3 ml of toluene and 0.01 ml of a 0.1 *M* solution of triethylamine in toluene. The tubes were sealed with tight-fitting stoppers, vigorously shaken for 30 s and placed in a heating cabinet at 60°C for 30 min. The remaining HFBA was evaporated under a stream of nitrogen and the residue was dissolved in ethyl acetate (0.01 ml) for GC analysis.

The instrument was a 5790A Hewlett-Packard gas chromatograph (Avondale, PA, U.S.A.) with a split–splitless capillary injection port and a ^{63}Ni electron-capture detection (ECD) system. The entire study was carried out with a cross-linked 5% phenylmethylsilicone, siloxane-deactivated fused-silica capillary column (Hewlett-Packard Ultraseries, 25 m×0.2 mm I.D). The column temperature was 250°C and argon containing 5% methane was used as the carrier gas. An aliquot of 0.001 ml of the residue dissolved in ethyl acetate was injected into

the chromatograph. The absolute retention times of the morphine-HFBA derivative and the nalorphine-HFBA derivative were 7.36 and 9.46 min, respectively.

RESULTS

Validity of the GC–ECD assay of morphine

Linear calibration curves were obtained when the peak-area ratio morphine-HFBA derivative/nalorphine-HFBA derivative was plotted against the concentration of morphine in spiked standard samples. Over the concentration range used (10–120 μg per 100 ml) the regression equation for spiked blood was $y=0.992x+0.74$ (correlation coefficient, $r=0.995$) and for spiked plasma $y=0.492x+0.028$ ($r=0.999$). The coefficient of variation (C.V.) for the analysis of morphine in blood was 6.73% at a level of 60 μg per 100 ml ($n=6$). The C.V. for the analysis of morphine in plasma was 5.24% at a level of 120 μg per 100 ml ($n=6$). Samples with a higher concentration than the highest standard were diluted to fall within the stated levels before repeating the analysis.

HFBA derivatives of morphine and nalorphine were found to be stable for 24 h at room temperature. They were found to undergo thermal decomposition when the chromatographic column temperature exceeded 265 °C.

Validity of the enzyme immunoassay test

In seven of the twenty cases the absorption in the spectrophotometer, when using plasma directly, was too high ($A>1500$–2000) to be reliable. For these cases only the value of reconstituted extract of plasma was used. For the rest of the cases, opiate concentrations obtained by the EMIT procedure for directly measured plasma and for the reconstituted extract of plasma were compared. To this end, we applied a Student's *t*-test to both series of values. The results did not show a statistically significant difference ($p=0.05$), indicating that we may use either plasma directly or a reconstituted extract of plasma. In all the twenty cases the plasma samples were found to contain morphine as is shown in the tables. Plasma samples from deaths not suspected to be heroin-related were analysed following the same procedure, and no positive response was obtained ($n=50$).

Recoveries of morphine

According to Predmore et al. [19], the yield of morphine after enzymatic hydrolysis is 80%. Using Sep-Pak C_{18} cartridges, the recoveries of morphine from spiked plasma and from spiked blood at a concentration of 10 μg per 100 ml were 90 and 78%, respectively. When using columns packed with Extrelut silica and chloroform–isopropyl alcohol the recovery of morphine from blood was 92% at a concentration of 10 μg per 100 ml.

Tables of data

The results are presented in three separate tables, depending on the circumstances of death. The first table (Table I) contains ten cases in which the subjects were found dead with a syringe still afixed in some part of the body. Five of them were found in bar toilets, three in their beds and two inside cars. The second table

TABLE I

CONCENTRATIONS OF MORPHINE, DEATH WITH SYRINGE AFFIXED

Abbreviations: M = male; F = female.

Case No.	Age	Sex	Concentration (μg per 100 ml)					
			Opiates	Total morphine in plasma	Total morphine in blood	Conjugated morphine in blood	Free morphine in blood	Other drugs found in blood
1	24	F	50	47	40	39.5	0.5	—
2	26	M	75	70	61	59.8	1.2	—
3	19	M	40	30	20	18.0	2.0	—
4	26	M	140	96	64	59.5	4.5	Ethanol: 0.24 g%
5	22	F	75	12	21	16.0	5.0	Ethanol: 0.28 g%
6	26	M	100	47	30	25.0	5.0	—
7	23	M	360	260	160	150.0	10.0	Alprazolam: 4 μg per 100 ml
8	25	M	95	30	53	40.0	13.0	—
9	30	M	120	66	40	26.0	14.0	7-Aminoflunitrazepam: 6 μg per 100 ml
10	26	M	450	180	260	240.0	20.0	Diazepam: 300 μg per 100 ml

TABLE II

CONCENTRATIONS OF MORPHINE, DEATH ON ARRIVAL AT THE HOSPITAL

Abbreviations: M = male; F = female; N.D. = not detected.

Case No.	Age	Sex	Concentration (μg per 100 ml)					
			Opiates	Total morphine in plasma	Total morphine in blood	Conjugated morphine in blood	Free morphine in blood	Other drugs found in blood
11	23	F	230	132	114	110.0	4.0	—
12	30	M	200	238	173	168.5	4.5	Cocaine: 18 Phenobarbital: 730
13	25	M	100	100	83	83.0	N.D.	—
14	26	M	80	45	12	12.0	N.D.	Phenobarbital: 1400
15	38	M	26	26	18	18.0	N.D.	—

(Table II) presents the results of five cases in which the subjects were found in stupor or coma, were taken to the hospital, but died before any clinical measures could be taken. Table III presents five cases of death in which a syringe was found near the cadaver, but in which it was difficult to establish the time of death.

DISCUSSION

Opiate concentrations obtained in plasma by the EMIT procedure should not be compared quantitatively to the concentrations of total morphine in plasma, because they have been obtained by two different techniques. One of these (EMIT) is only semiquantitative, and shows cross-reactivity with possibly present minor metabolites of heroin. The use of this immunoassay is recommended

TABLE III

CONCENTRATIONS OF MORPHINE, SYRINGE RECOVERED ALONGSIDE THE BODY

Abbreviations: M = male; F = female; N.D. = not detected.

Case No.	Age	Sex	Concentration (μg per 100 ml)					
			Opiates	Total morphine in plasma	Total morphine in blood	Conjugated morphine in blood	Free morphine in blood	Other drugs found in blood
16	29	M	1300	440	144	88.5	55.5	Phenobarbital: 330 Methaqualone: 53.8 Cocaine: 212 Diazepam: 100
17	27	M	240	110	89	89.0	N.D.	Phenobarbital: 200 Cocaine: 183 Lignocaine: 92 Bencydamine: 59
18	24	M	50	37	34	34.0	N.D.	Diazepam: 9
19	25	M	47	47	18	18.0	N.D.	—
20	30	M	30	30	5	5.0	N.D.	Desmethyldiazepam: 100 Oxazepam: 30

only as a rapid means for discarding negative samples. The presence of morphine can be confirmed later.

The comparison between total morphine concentrations in plasma and in whole blood shows higher values for the former in all but three cases, suggesting that the total morphine concentration is higher in plasma than in blood. Unfortunately, the analysis of free morphine in plasma was not possible, because the amount of plasma required exceeded the amount of sample available. The concentrations found for total morphine in whole blood are in agreement with the data published by others [9,11,12,20].

Conjugated morphine in blood was detected in all cases, while free morphine appeared in thirteen samples (all ten samples of the first group, two of the second and one of the third). The measured concentrations of conjugated morphine are higher than those of free morphine, but no mathematical relationship could be established between the two series of data. The main significance of the conjugated morphine is its presence in all the cases, which may explain the unexpected absence of morphine in previously published cases [2,3,11,12,15,16]. The presence of conjugated morphine in blood is the only possibility of heroin-related death when no free morphine is detected in blood.

The concentrations of free morphine in samples of the first group are below the concentrations accepted as fatal [14], although they are in agreement with data from other authors who described fatal cases with lower concentrations [16,25]. The concentrations in the second table are in the same range as those of the first group for free morphine, while the only positive case in the third table shows the highest value of all. Hence, there appears to be little correlation between the concentration of free morphine and the duration of life after the heroin injection. However, there is a strong correlation between the duration of life after heroin injection and the presence of free morphine in blood. Thus, we suggest

that the presence of free morphine in blood indicates a recent heroin injection, whereas conjugated morphine in blood indicates sustained life over a comparatively longer period.

In this study, separately measured concentrations of free and conjugated morphine in blood are compared. The results explain some of the previously published results and provide additional information to aid in the diagnosis of death due to heroin. An enzyme immunoassay for plasma is especially useful when it can be applied as a rapid means of assessing the presence of opiate derivatives in blood.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Miss A. Rodriguez and Mrs. K. Jarrell for their careful review of this manuscript.

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