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Immunoaffinity extraction of morphine, morphine-3-glucuronide and morphine-6-glucuronide from blood of heroin victims for simultaneous high-performance liquid chromatographic determination

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Abstract

The development of an immunoaffinity-based extraction method for the determination of morphine and its glucuronides in human blood is described. For the preparation of an immunoadsorber, specific antisera (polyclonal, host: rabbit) against morphine, morphine-3-glucuronide and morphine-6-glucuronide were coupled to 1,1′-carbonyldiimidazole-activated tris-acrylgel and used for immunoaffinity extraction of morphine and its glucuronides from coronary blood. The resulting extracts were analysed by HPLC with native fluorescence detection. The mean recoveries from spiked blood samples were 71%, 76% and 88% for morphine, morphine-3-glucuronide and morphine-6-glucuronide, respectively. The limit of detection was 3 ng/g blood and the limit of quantitation was 10 ng/g blood for all three analytes. The results of the analysis of coronary blood samples from 23 fatalities due to heroin are presented.

Keywords: Morphine; Morphine glucuronide

1. Introduction

After the intake of heroin (3,6-diacetyl morphine), a rapid deacetylation to 6-monoacetyl morphine (6-MAM) and finally to morphine (M) takes place which is caused by the serum cholinesterase acylcholine acylhydrolase [1–4]. The halflife of heroin is 5–9 min, that of 6-MAM is between 38–45 min whilst that of M varies between 90 and 180 min. The conjugation of M to activated uridine 5′-diphosphoglucuronic acid (UDPGA) in the liver and intestines via the phenolic hydroxyl group results in the main metabolite morphine-3-β-D-glucuronide (M3G), a pharmacologically inactive metabolite with opiate antagonistic effects. The glucuronidation via the secondary alcoholic hydroxyl group results in the minor metabolite morphine-6-β-D-glucuronide (M6G), a metabolite with questionable pharmacological activities. Recent studies have shown that when administered by different routes, M6G may pass the blood–brain barrier and induce analgesic and respiratory depressing activities [5–14]. But the ability of M6G to pass the blood–brain barrier has been questioned since no analgesic activity was observed when M6G was administered intravenously in humans [15,16].

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In forensic cases of heroin intake the molar ratio of morphine glucuronides to M from blood can be used for an estimation of the time elapsed between the last intake and the time when the blood sample was taken or time of death [17,18]. For this reason the simultaneous determination of M and morphine glucuronides can be important in forensic samples.

Methods using solid-phase extraction and HPLC quantitation with UV detection [19,20], electrochemical detection [21–24], native fluorescence detection [17,25–29] or LC–MS [20,30,31] have been described. A simple and efficient technique for sample purification and concentration from biological samples is immunoaffinity chromatography using immobilised antibodies which has already been successfully applied for the extraction of drugs of abuse such as tetrahydrocannabinol [32], flunitrazepam [33], or lysergic acid diethylamide (LSD) [34–36]. In extraction experiments of M using an immunoadsorber made from commercially available antiM antisera, morphine glucuronides could also be extracted [37].

These results gave rise to the development of antisera specific against M, M3G and M6G [38]. In the following, the extraction of M and its glucuronides by affinity chromatography with these antisera is described. The matrix chosen for the immobilisation of antibodies was 1,1′-carbonyldiimidazole-activated trisacrylgel (CDIT) which enables a simple and rapid binding by the formation of stable and uncharged urethan bonds via the α- and ϵ-amino groups of the antibodies.

2. Experimental

2.1. Reagents and chemicals

Reacti-Gel™ GF-2000 (=CDIT), bovine serum albumin (BSA) and bicinchoninic acid (BCA) protein assay reagent: Pierce (Rockford, IL, USA). Visking dialysis tube: Serva (Heidelberg, Germany). M3G and M6G: Sigma (Deisenhofen, Germany). Morphine hydrochloride trihydrate: Merck (Darmstadt, Germany). All other chemicals were of analytical grade. Acetonitrile was of HPLC grade.

Phosphate buffered saline pH 7.4 (PBS) contained 0.137 mol NaCl, 0.008 mol $Na_2HPO_4 \cdot 2H_2O$, 0.001 mol KH_2PO_4, 0.003 mol KCl and 0.003 mol NaN_3/l H_2O. Saturated ammonium sulphate solution was made by dissolving 0.6 mol $(NH_4)_2SO_4$ in 100 ml H_2O and was adjusted to pH 7.0 by the addition of NaOH. The coupling buffer solution contained 0.1 mol $Na_2B_4O_7 \cdot 10H_2O$/l H_2O, adjusted to pH 8.5 with a solution of 0.1 mol KH_2PO_4/l H_2O. The blocking buffer solution consisted of 1.0 mol ethanol amine/l H_2O, adjusted to pH 9.0 with H_3PO_4. The eluent for affinity chromatography consisted of 100 ml of a solution of 0.2 mol glycine and 0.05 mol NaCl/l H_2O, adjusted to pH 2.0 with HCl and 900 ml methanol.

2.2. Preparation and pretreatment of polyclonal antisera

The production of specific polyclonal antisera against M, M3G and M6G (host: rabbit) has been described previously [38]. The purification was carried out by a two-step procedure.

1. To separate the antibodies directed against the carrier protein BSA, the raw antisera were incubated with BSA. For all antisera tested, 10 μl of a 0.25% BSA solution in PBS was sufficient to precipitate all antibodies against BSA. Accordingly 1 ml of BSA/PBS solution was dropped slowly under stirring into 25 ml raw antisera. These mixtures were incubated at 4°C overnight and the precipitated proteins separated by centrifugation for 30 min at 3000 *g*.
2. The supernatants were used for the isolation of protein fractions by ammonium sulphate saturation [39]. A saturated ammonium sulphate solution pH 7.0 was dropped to the supernatants under stirring up to 50% saturation. These mixtures were stored at 4°C overnight and the precipitates separated by centrifugation for 30 min at 3000 *g*. The resulting pellets were dissolved in 15 ml PBS and three times dialysed against 1000 ml PBS for 12 h at 4°C. The protein content of raw antisera and antisera after pretreatment was determined spectrophotometrically using the BCA protein assay reagent.

2.3. Preparation of immunoaffinity columns

The immobilisation of antibodies using CDIT was performed according to the manufacturers recommendation and carried out in three separated batches.

- Portions of each 5 ml CDIT were washed with water and coupling buffer whereby the volume increased by a factor of 1.8
- each portion of washed gel was mixed with 10 ml coupling buffer and an amount of pretreated antiserum equivalent to 100 mg protein (3.29 ml of antiM, 3.50 ml antiM3G or 3.46 ml antiM6G) and rotated for 30 h at room temperature
- the coupling products were centrifuged for 10 min at 2000 *g* and the supernatants were collected for the determination of protein loading using BCA protein assay reagent
- the pellets were washed with blocking buffer and rotated with 10 ml blocking buffer for 3 h at room temperature to block the remaining active groups of CDIT
- the coupling products were washed with PBS until the pH was 7.4 and were now ready for use

For the determination of the loading capacities of the different coupling products, aliquots of 1 ml coupling product were introduced into glass columns with a total volume of 20 ml. For the extraction of M, M3G and M6G from blood samples, the three different coupling products were mixed and columns were prepared by filling 6 ml of this mixture into a glass column.

2.4. Immunoaffinity extraction of M, M3G and M6G

2.4.1. Determination of loading capacity

To determine the loading capacity of the three immunoaffinity matrices, columns were prepared by filling 1 ml of antiM matrix, antiM3G matrix or antiM6G matrix into separate glass columns and 1 ml of analyte solutions in the concentrations 100, 200, 300, 400 and 500 ng/ml in PBS were extracted. The extraction procedure was carried out according to the following scheme:

- 1.0 ml analyte solution was placed on an immunoaffinity column which was then sealed and rotated around the longitudinal axis using a laboratory shaker (REAX 2, Heidolph) for 60 min at room temperature
- the solution was run through, 10 ml eluent was placed in the column which was again rotated for 60 min at room temperature
- the eluate was collected, evaporated to dryness in vacuum (10^4 Pa, 60°C) and dissolved in 1 ml demineralized water. This solution was used for the HPLC determination of M, M3G and M6G.

The regeneration of the columns was achieved by rinsing with PBS until the pH of the eluate was 7.4.

2.4.2. Extraction of M, M3G and M6G from blood

For the simultaneous extraction of M, M3G and M6G from blood the three different immunomatrices were mixed in the ratio 1:1:1 and 6 ml of this mixture was filled into a glass column. For validation of the method, drug-free postmortem human blood samples were spiked with M, M3G and M6G in the range 0, 10, 50, 250, 500 and 1000 ng/g. The extraction of spiked blood samples and blood of heroin addicts was performed according to the following scheme:

- 0.5 g blood was mixed with 0.5 ml PBS and centrifuged at 6000 *g* for 10 min
- the supernatant was placed on an immunoaffinity column which was then sealed and rotated around the longitudinal axis for 60 min at room temperature
- the sample was run through and the column was washed with four aliquots of 5 ml demineralized water
- 5 ml of the eluent was run through the column and collected
- 10 ml of the eluent was mixed with the content of the column which was again sealed and rotated for 60 min at room temperature; the eluent was passed through and collected
- another 5 ml of the eluent was run through the column and collected
- the combined collected eluent was evaporated to dryness in vacuum (10^4 Pa, 60°C) and reconsti-

tuted in 0.5 ml demineralized water. This solution was used for the HPLC determination of M, M3G and M6G.

The columns were regenerated again as described.

2.5. HPLC conditions

A Hewlett-Packard Series II 1090 liquid chromatograph and a Hitachi Merck F-1050 fluorescence spectrophotometer with a mercury-xenon lamp and a 12 μl flow cell were used. The excitation wavelength was 235 nm and emission was recorded at 345 nm. Chromatographic separation was performed with a LiChrospher 60 RP-select B (5 μm, 250×4 mm) analytical column (Merck). The mobile phase consisted of 92.5% 10 m*M* KH_2PO_4, 2 m*M* heptane sulfonic acid adjusted to pH 2.5 with H_3PO_4 and 7.5% acetonitrile.

3. Results and discussion

3.1. Antisera and pretreatment of antisera

For the production of immunoaffinity matrices by immobilisation of antibodies, three different polyclonal antisera (antiM, antiM3G and antiM6G) were chosen which were very specific for the recognition of M, M3G or M6G [38]. The high specificity of these antisera necessitated the formation of three different affinity matrices, each individual for the analyte, the antiserum was created against.

The incubation of the raw antiM, antiM3G and antiM6G antisera with BSA, the carrier protein to which the haptens were coupled for the immunisation of rabbits, led to the precipitation of antibodies directed against BSA. These antiBSA antibodies always occur when BSA is used as carrier protein for the immunisation of animals. The removal of these antibodies which are not directed against M or morphine glucuronides is necessary because the immobilisation of antibodies by linking them to a matrix is a process in which a nonspecific binding of protein to an activated surface of a chemical stable matrix takes place. In order to obtain an immunoadsorber with high loading of specific antibodies, it is necessary to remove nonspecific antibodies before coupling is performed.

The protein fractions of the sera were isolated by precipitation with ammonium sulphate, reconstituted in PBS and dialysed. The protein concentrations of crude antisera before and after pretreatment, determined spectrophotometrically are listed in Table 1. The protein concentration decreased by more than 50% in all three cases.

3.2. Immobilisation of antibodies

For the immobilisation of antibodies, CDIT is suitable because the reaction of α- and ε-amino groups of proteins with CDIT (shown in Fig. 1) leads to stable and uncharged urethan bonds with no ion-exchange effects. Experiments on immobilisation of antibodies using commercial available polysaccharide matrices like cyanogen bromide-activated Sepharose lead to immunoadsorbers with lower reusability in comparison with CDIT.

In order to establish suitable conditions for the immobilisation of pretreated antisera, the coupling procedure was performed in different ratios of protein to CDIT in the range from 5 mg protein/ml CDIT to 20 mg protein/ml CDIT. The results of this study, which are presented in Fig. 2, show that with increasing amounts of protein in the reaction mix-

Table 1
Protein contents of crude antisera and antisera after pretreatment

Antiserum	Protein content (mg/ml)		Ratio of protein content (antiserum after pretreatment to crude antiserum)
	Crude antiserum	Antiserum after pretreatment	
AntiM	64.0	30.4	0.48
AntiM3G	63.1	28.6	0.45
AntiM6G	63.8	28.9	0.45

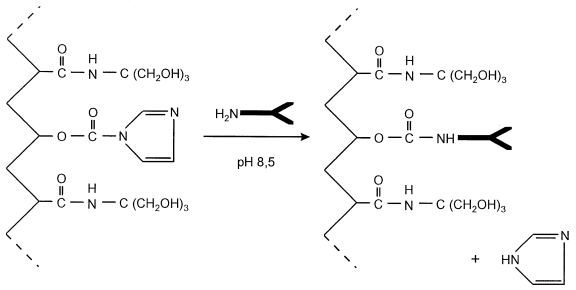

Fig. 1. Immobilisation of antibodies by coupling them to 1,1′-carbonyldiimidazole-activated trisacrylgel via their α- or ϵ-amino groups.

ture, the absolute protein loading of CDIT increased proportionally whereas the efficiency of the protein conjugation, expressed as the percentage of bound protein decreased. For the later studies a low coupling efficiency was acceptable to obtain a high protein loading (a sufficient pool of antisera was available). The protein loading of the immunoadsorber used for the extraction of M, M3G and M6G are listed in Table 2.

3.3. Determination of the column capacity

The method used for the definition of the column capacity is based on the determination of recovery according to the different amounts of analyte given on the column. Therefore solutions with increasing amounts of analyte have to be extracted. The limit of

Table 2
Coupling efficiency and protein loading of immunoadsorbers

Pretreated antisera	20 mg protein/ml CDIT used for coupling	
	Coupling efficiency (%)	Protein loading (mg/ml CDIT)
AntiM	65	13.0
AntiM3G	68	13.4
AntiM6G	66	13.3

column capacity is reached with the quantity of analyte which leads to a decreasing recovery. This method has been employed to characterise the immunoadsorbers for the extraction of M, M3G and M6G. The findings of this procedure are shown in Fig. 3. In all three cases the recovery decreased in the range of 300 ng analyte per ml of immunoadsorber. So the column capacity of all three immunoaffinity matrices was assumed to be 250 ng analyte per ml of immunoadsorber. For the extraction of M, M3G and M6G from spiked blood samples or blood samples from drug addicts, the three different immunoadsorbers were mixed in the ratio 1:1:1 and columns were filled with a volume of 6 ml of this mixture. Using these columns with a capacity of 500 ng for M, M3G and M6G the desired concentration range up to 1000 ng/g of each analyte can be covered when 0.5 g blood are extracted. The columns can be reused 20 times. When they are used more often a decreasing column capacity is detectable.

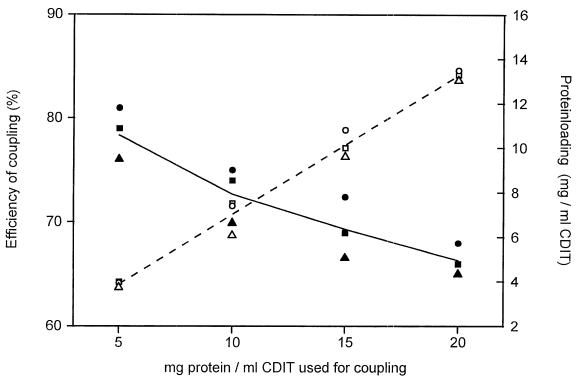

Fig. 2. Correlation between efficiency of coupling and protein loading of 1,1′-carbonyldiimidazole-activated trisacrylgel (CDIT) when different ratios of protein: CDIT were used.

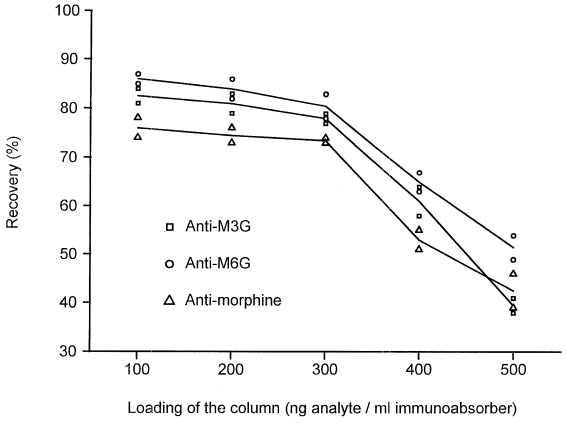

Fig. 3. Determination of the column capacity via the recoveries for morphine, M3G and M6G.

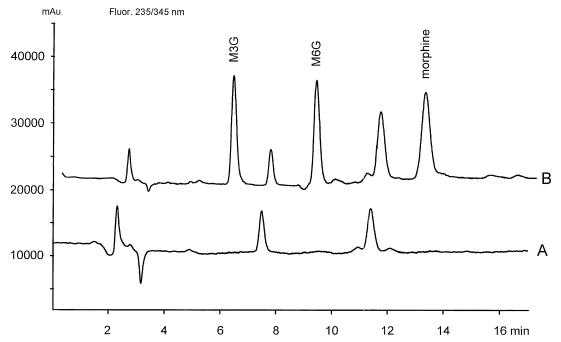

Fig. 4. HPLC chromatograms of a drug-free postmortem blood sample (A) and a blood sample spiked with 250 ng/g morphine, M3G and M6G (B).

3.4. Validation of the method

3.4.1. Specificity

The specificity of the method was examined by a comparison of the chromatogram of a drug-free blood sample with the chromatogram of a blood sample spiked with 250 ng M, M3G and M6G/g (Fig. 4). There are no interfering peaks in the chromatogram of blank blood at the retention times of M, M3G or M6G although a nonspecific binding could be observed.

3.4.2. Recovery

For recovery experiments blood was spiked in four different concentration levels (50, 250, 500 and 1000 ng/g) with M, M3G and M6G and from each level three samples were analysed. The recovery was calculated from the peak areas of the spiked blood samples and the peak areas of standard solutions in the same concentration ranges (Table 3). One disadvantage of the extraction method is the decrease of recoveries in the upper concentration range in comparison with the lower range. This effect may be the result of the limited column capacity.

Table 3
Recovery of morphine, M3G and M6G

Concentration (ng/g)	Recovery (%)		
	Morphine	M3G	M6G
50	76±4	84±4	88±3
250	72±4	79±4	90±3
500	68±3	71±3	88±4
1000	66±2	70±4	84±3

3.4.3. Linearity

The linearity of the results was verified in the concentration range 10 to 1000 ng M, M3G and M6G/g blood. This range covers the concentrations expected in authentic samples. At each concentration level (10, 50, 250, 500 and 1000 ng/g) two spiked blood samples were analysed. The linear regression of the calibration curve of M is described with the function $y=6.99x+8181$ and the correlation coefficient $r=0.9992$, the curve of M3G with $y=702x+10030$ and $r=0.9994$ and the curve for M6G with $y=831x+8009$ and $r=0.9996$.

3.4.4. Precision and accuracy

Blank blood samples were spiked in four concentration levels (50, 250, 500 and 1000 ng/g) with M, M3G and M6G. For the determination of precision these samples were extracted and analysed three times in one day (intra-assay variance) and three times each on three different days (inter-assay variance). In our studies intraassay and inter-assay variances were acceptable. The coefficients of variation (C.V.) were less than 10% (Table 4). The accuracy for M in the range 96 to 105%, for M3G between 95 and 108% and for M6G between 95 and 107% was also satisfactory. The limit of detection (signal-to-noise ratio of 3) was approximately 3 ng/g blood and the limit of quantitation (signal-to-noise ratio of 10) about 10 ng/g blood for all three analytes.

3.5. Blood samples from heroin victims

Recent studies have shown that in cases of fatal heroin overdose the concentrations for M and its glucuronides in blood may be used for an estimation of the time elapsed between the last intake of heroin and the occurrence of death [18]. The data base for this estimation were pharmacokinetic studies of the metabolism of M, administered by intravenous injection [17]. The survival time ranges probably between 2 and 8 h when the molar ratio of M6G/M is above 3 and is less than 30 min when the ratio is below 1. The above described method was applied for the determination of M, M3G and M6G from

Table 4
Precision and accuracy for the determination of morphine, M3G and M6G

	Concentration added (ng/g blood)											
	Morphine				M3G				M6G			
	50	250	500	1000	50	250	500	1000	50	250	500	1000
	Determined concentration (ng/g blood)											
Series 1 (n=3)	49	255	507	993	48	254	509	980	48	256	510	1013
Series 2	52	254	510	1019	54	257	490	1007	48	245	488	976
Series 3	48	241	506	983	49	242	508	986	53	244	508	980
	Precision (C.V. %), arithm. mean value											
Series 1 (n=3)	9.28	2.93	2.61	2.26	9.43	4.71	4.36	5.33	9.90	3.32	1.14	2.26
Series 2	9.40	2.98	2.74	3.20	8.00	3.36	3.31	3.63	9.43	2.41	2.26	0.52
Series 3	9.00	2.93	2.92	0.60	8.33	1.86	2.17	0.95	7.71	3.00	3.04	0.50
	Accuracy (%), arithm. mean value											
Series 1 (n=3)	98	102	101	99	95	101	102	98	97	102	102	101
Series 2	105	102	102	102	108	103	98	101	95	98	98	98
Series 3	96	96	101	98	99	97	102	99	107	98	102	98

blood samples of 23 heroin victims. Coronary blood samples were collected during autopsy, immediately frozen and stored at −20°C until examination. The results of these measurements are listed in Table 5. A typical HPLC chromatogram (case no. 17) is shown in Fig. 5. In all cases the intravenous abuse of

Table 5
Concentration of morphine and morphine glucuronides in cases of fatal heroin doses

Case	Coronary blood (ng/g)			Molar ratio		
	M	M3G	M6G	M3G/M	M6G/M	M3G/M6G
1	97	11	n.d.[a]	0.07	–	–
2	491	24	<10[b]	0.03	<0.01	>2.40
3	263	37	<10[b]	0.09	<0.02	>3.70
4	1110	163	40	0.09	0.02	4.08
5	400	77	22	0.12	0.03	3.50
6	85	28	<10[b]	0.20	<0.07	>2.80
7	93	53	11	0.35	0.07	4.82
8	291	225	52	0.48	0.11	4.33
9	240	162	43	0.42	0.11	3.77
10	431	484	87	0.69	0.12	5.56
11	167	164	34	0.61	0.13	4.82
12	298	174	62	0.36	0.13	2.81
13	129	117	32	0.56	0.15	3.66
14	44	60	13	0.84	0.18	4.62
15	247	281	80	0.70	0.20	3.51
16	228	372	79	1.01	0.21	4.71
17	446	538	161	0.75	0.22	3.34
18	33	61	13	1.14	0.24	4.69
19	254	383	104	0.93	0.25	3.68
20	390	750	168	1.19	0.27	4.46
21	94	309	53	2.03	0.35	5.83
22	<10[b]	80	29	>4.95	>1.79	2.76
23	<10[b]	130	40	>8.04	>2.47	3.25

[a] n.d.=not detectable.
[b] =analyte detectable but below limit of quantitation.

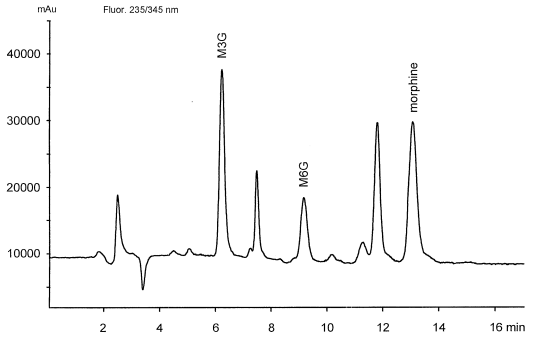

Fig. 5. HPLC chromatogram of case 17 with 446 ng morphine, 538 ng M3G and 161 ng M6G/g coronary blood.

heroin was known. In case 1 the molar quotient of M6G/M could not be calculated because M6G was not detectable. For cases 2 to 21 the survival time after the last intake of heroin must have been less then 30 min. Only in the cases 22 and 23 the molar ratios of M6G/M are above 1 which means that the time period elapsed since the last heroin dose has been longer than 30 min. The circumstances of discovery of the bodies and the autopsy findings confirmed these results.

A typical example for a short survival time is case 2. The lethal heroin dose was injected intravenously to the 37 year old male victim by a friend because the victim was too alcoholized (2.51‰ ethanol in femoral blood) to do it by himself. He collapsed soon after the injection so that the immediately called emergency doctor found him dead only 15 min after the injection. The autopsy 28 h after the occurrence of death showed an aspiration of stomach content and the signs of the sudden death.

In case 23, an example with prolonged survival time the body of a 33 year old man was found dead on the ice-sheet of a frozen canal. The autopsy 23 h later and histological findings showed that the man died of cold.

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