Int J Legal Med (1997) 110 : 226–229

SHORT COMMUNICATION

J. Beike · H. Köhler · G. Blaschke

Antibody-mediated clean-up of blood for simultaneous HPLC determination of morphine and morphine glucuronides

Received: 10 November 1996 / Received in revised form: 19 February 1997

Abstract For the interpretation of the concentration of morphine in blood samples of heroin consumers information about the concentration of the analgesic active morphine metabolite morphine-6-glucuronide is very important. Thus a simple but specific clean-up procedure based on immuno-affinity chromatography is presented for the extraction of morphine, morphine-3-glucuronide and morphine-6-glucuronide from whole blood in cases of fatal heroin overdose. The preparation of the immunoabsorber by immobilization of antibodies against morphine-3-BSA and morphine-6-KLH with carbonyldiimidazole-activated trisacrylgel is described. The separation of the extracts is achieved by HPLC using native fluorescence detection. The limits of detection for this method are 10 ng for morphine and morphine glucuronides/g blood. The results for the concentration of morphine and morphine glucuronides in blood from seven cases of heroin overdose are presented. By calculating the quotients for the concentrations of morphine-6-glucuronide/morphine the time elapsed since the last intake of heroin is estimated.

Key words Morphine glucuronides · Antibody-mediated clean-up · Immunoabsorber · HPLC native fluorescence detection

Introduction

Following ingestion, 3,6-diacetyl morphine (heroin) is rapidly deacetylated to 6-monoacetyl morphine and finally to morphine (M) [1–4]. M is primarily metabolized to morphine-3-glucuronide (M3G), and to a lesser extent to morphine-6-glucuronide (M6G), morphine-3-etherealsulfate and normorphine [5, 6]. The conjugation to glucuronic acid in the liver and intestines results in approximately 50% M3G and 10% M6G. While M3G is pharmacologically inactive and an opiate antagonist, M6G is pharmacologically active and showed a 15–20 fold higher analgesic potency in animal experiments (rats) than M itself [7–11]. For these reasons the simultaneous determination of M and morphine glucuronides (MGs) in cases of fatal overdose is necessary in order to interpret the results better.

Methods using solid-phase sample purification and quantification of M and MGs by HPLC with UV detection [12–14], electrochemical detection [15–19] and native fluorescence detection [20–23] have been described. A very simple and practical technique for sample purification and concentration from complex matrices is the use of immobilized antibodies as an immunoabsorber [24–26]. In the method described in this paper antibodies against M were immobilized by coupling to carbonyldiimidazole-activated trisacrylgel to create an immunoabsorber for sample purification. The clean-up of whole blood samples from victims of heroin overdose for HPLC quantitation with native fluorescence was performed using this material because it combines the specificity of an antibody reaction and the selectivity of the HPLC separation.

Material and methods

Reagents and chemicals

Morphine-3-β-D-glucuronide and morphine-6-β-D-glucuronide were purchased from Sigma (Deisenhofen, Germany), morphine hydrochloride trihydrate, codeine hydrate and brij 35 (polyoxyethylene dodecyl ether) from Merck (Darmstadt, Germany) and dihydrocodeine hydrogen tartrate from Knoll AG (Ludwigshafen, Germany). Reacti-Gel GF-2000 (= 1,1′-carbonyldiimidazole-activated trisacrylgel GF-2000 = RG) and BCA protein assay were purchased from Pierce (Rockford, Il., USA).

The antiserum against morphine-3-BSA was obtained from Sigma Immuno Chemicals (Deisenhofen, Germany) and against morphine-6-KLH from Affiniti (Nottingham, UK).

J. Beike · H. Köhler (✉)
Institut für Rechtsmedizin, Westfälische Wilhelms-Universität, von Esmarchstrasse 86, D-48149 Münster, Germany
Fax: +49 (251) 8355 158

G. Blaschke
Institut für Pharmazeutiche Chemie, Westfälische Wilhelms-Universität, Hittorfstrasse 58, D-48149 Münster, Germany

Stock solutions of 1 mg/ml M3G and 1 mg/ml M6G were prepared in water and 1 mg/ml of M, codeine and dihydrocodeine were prepared in methanol. Working standards for HPLC were prepared in the range of 5 ng/ml–1000 ng/ml by diluting the stock solutions with HPLC eluent.

Drug-free postmortem human blood was spiked with M, M3G and M6G in the range from 5 ng/g to 1000 ng/g.

Concentrated phosphate-buffer saline (PBS, pH 7.4) contained 0.1368 M NaCl, 0.0081 M Na_2HPO_4, 0.0015 M KH_2PO_4, 0.0027 M KCl, 0.031 M NaN_3. The coupling buffer solution contained 0.1 M $Na_2B_4O_7$ adjusted to pH 8.5 with boric acid. The blocking buffer solution consisted of 1.0 M ethanolamine adjusted to pH 9.0 with H_3PO_4 and acetate buffer (pH 4.0) contained 0.1 M sodium acetate.

Preparation of immunoaffinity columns

Aliquots (10 ml) of Reacti-Gel GF-2000 (RG) were washed with water and coupling buffer whereby the volume of gel increased to 18 ml. This gel was divided into two equal portions, each portion was mixed with 10 ml coupling buffer and 1 ml of antiserum against either M3-BSA or M6-KLH and mixed on a rotator for 30 h at room temperature. The coupling products were centrifuged for 5 min at 2000 g and the supernatants were used for determination of protein loading. In order to block the remaining active groups of RG the gel was washed with blocking buffer, mixed with 10 ml of blocking buffer and rotated for 2 h at room temperature. The products were washed with three cycles of alternating pH using acetate buffer and PBS. Finally both coupling products were mixed in PBS and 3 ml of this mixture was placed in sealed glass columns.

The efficiency of protein conjugation to RG was determined from the protein concentration of the antisera before the coupling procedure and in the supernatants after coupling. The concentration of protein was determined spectrophotometrically using BCA protein assay.

To determine the column capacity increasing amounts of M, M3G and M6G were extracted from PBS solution using columns with a volume of 1 ml RG.

Extraction procedure

Aliquots (1 g) of blood from heroin victims or spiked blood samples were weighed in a centrifuge tube, made up to 5 ml with PBS, vortexed and centrifuged at 3000 g for 10 min. Of the supernatant 2 ml (= 400 mg blood) was placed on an affinity column which was then sealed and rotated for 20 min. The column was washed with 20 ml water containing 1 mg brij 35. The antibody-bound M and MGs were eluted by treatment of the column with 20 ml of a methanol/water mixture (10:1). The eluate was evaporated to dryness in a vacuum and reconstituted in 400 µl mobile phase solvent A for HPLC analysis.

Regeneration

To avoid the risk of cross-contamination the column was washed again with 20 ml of eluent. The regeneration was achieved by washing the column with 20 ml PBS, followed by rotating for 5 min with 10 ml PBS and another washing step with 20 ml PBS. In this way the columns could be reused at least 15 times without loss of capacity.

HPLC-conditions

A Hewlett Packard Series II 1090 liquid chromatograph and a Hitachi Merck F-1050 fluorescence spectrophotometer with mercury-xenon lamp and a 12-µl flow cell were used.The excitation wavelength was 240 nm and emission was recorded at 340 nm. Chromatographic separation was performed with a LiChrospher 60 RP-select B (5 µm, 250 × 4 mm) analytical column (Merck, Darmstadt, Germany).

Sample volumes of 20 µl were subjected to HPLC and two solvents were used for gradient elution. Solvent A consisted of 10 mM KH_2PO_4 and 2 mM heptane sulfonic acid, adjusted to pH 3.0 with H_3PO_4. Solvent B was prepared by adding 250 ml acetonitrile to 250 ml of solvent A. Gradient elution was carried out at 40° C at a constant flow-rate of 1 ml/min as follows: initial conditions were 10% solvent B for 5 min, followed by a gradient of 10–30% solvent B over 15 min. After injection of the blood samples solvent B was used at 100% for 2 min to wash the column.

Results and discussion

Antibody-mediated clean-up

In this clean-up procedure immobilised antibodies were used to extract opiates, especially MGs from blood. For this method the chosen antisera showed a conveniently high cross-reactivity against M3G and M6G. It was not necessary to use antisera which have a specificity for certain opiates because the separation of the extracted opiates was attained in the subsequent HPLC procedure. Antibodies against M3-BSA show a high cross-reactivity against M3G because the steric structure of the immunogen excludes the conjugated glucuronic acid as part of the epitope. For the same reason there is a high cross-reactivity of antibodies produced with M6-KLH against M6G and therefore both antisera were used for the preparation of immuno affinity-columns.

A simple method has been chosen for the preparation of blood samples for extraction. It allows the separation of solid blood components so that the columns only contained those components which could be removed with brij solution and water. The extraction results were equivalent to those achieved using ultraturax or ultra sonic.

The elution of bound M and MGs was performed by a reversible change of the quaternary protein structure using a methanol/water mixture. This solution is a suitable solvent for M and MGs and can be readily evaporated to dryness under mild conditions before reconstitution in the HPLC eluent. This extraction procedure with a high level of purification results in chromatograms with no interfering peaks. Figure 1 shows a typical HPLC separation after antibody-mediated clean-up.

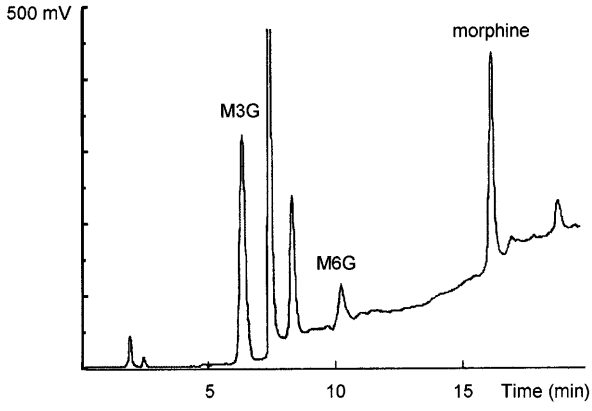

Fig. 1 Chromatogram of case no. 2: 401 ng M3G, 44 ng M6G and 237 ng morphine/g blood

Table 1 Efficiency of the antiserum conjugation to RG, expressed as percentage of bound protein

Antiserum against	Protein content of anti-serum	Protein used for coupling to 5 ml RG	Protein bound to RG		Protein loading per ml of RG
M3-BSA	67 mg/ml	67 mg	38 mg	57%	7.6 mg
M6-KLH	76 mg/ml	191 mg	120 mg	63%	24.0 mg

Table 2 Column capacity

	M	M3G	M6G	Codeine	Dihydo-codeine
ng/ml RG in PBS	314	277	254	186	160
nmol/ml RG in PBS	1.10	0.60	0.55	0.62	0.53

During the regeneration procedure the columns were washed a second time with the methanol/water mixture to avoid the risk of cross-contamination. M and MGs were not detectable in this eluate demonstrating that the elution was complete.

Conjugation efficiency

The efficiency of the antiserum conjugation to RG was expressed as a percentage of bound protein. Therefore the protein content of antisera before coupling and the protein content of the supernatants after coupling were determined using a BCA protein assay. The results presented in Table 1 show that nearly the same proportion of protein from the antisera is bound to RG but the protein loading of the coupling products is very different. The protein loading of antiserum against M3-BSA could perhaps be increased by using higher amounts of antiserum.

Column capacity

For the determination of the capacity, immuno affinity columns with a bed volume of 1 ml mixed coupling products were prepared. The results of extracting solutions of 1000 ng M, MGs, codeine or dihydrocodeine in 5 ml PBS with these columns are illustrated in Table 2. For comparison the molar column capacity must be considered. As expected the binding of 1.10 nmol M to 1 ml of immuno absorber is the highest. The decreasing cross-reactivity of analytes against chosen antisera is proportional to the binding rate which declines to 0.53 nmol for dihydrocodeine.

Spiking studies

Recovery experiments were carried out on drug-free postmortem human blood spiked up to a level of 1000 ng/g. The recovery was 73% for M, 82% for M3G and 74% for M6G. The standard curves for M, M3G and M6G are linear up to 1000 ng/g. The limits of detection for this method are 10 ng for M, M3G and M6G/g blood (signals corresponding to 3 times the noise level).

Table 3 Concentration of M and MGs in cases of fatal heroin doses

Case no.	M3G ng/g blood	M6G ng/g blood	M ng/g blood	Quotients (mol/mol)	
				M3G/M	M6G/M
1	476	53	93	3.16	0.35
2	401	44	237	1.04	0.11
3	167	12	201	0.51	0.04
4	126	19	152	0.51	0.08
5	343	42	593	0.36	0.04
6	122	10	331	0.23	0.02
7	209	44	769	0.16	0.03

Heroin-related deaths

Recent studies [27] show that in cases of fatal heroin overdoses the time elapsed since the last intake of heroin can be estimated by calculating quotients for the concentration of M3G/M and M6G/M. In fatal overdoses the quotient M6G/M is below 2, in cases with extremely short time periods (less then 1 h) between intake and death the quotient can be below 1.

The method described was used to analyse coronary blood from seven cases of heroin related-deaths with unknown time elapsed since the last intake of heroin. In all seven cases the abuse of heroin by iv-injection was known. The chromatogram in Figure 1 shows a typical HPLC separation after antibody-mediated clean-up. The results of this determination which are shown in Table 3 should give information about the period of time between the intake of the fatal heroin dose and the moment when death occurred. In all seven cases the quotients M6G/M were below 1 so it is probable that all of these heroin victims died within a 1-h period after intake of the fatal dose.

References

1. Wright CL (1942) The deacetylation of heroin and related compounds by mammalian tissue. J Pharmacol Exp Ther 75:328–337
2. Nakamura GR, Thornton JL, Noguchi TT (1975) Kinetics of heroin deacetylation in aqueous alkaline solution and in human serum and whole blood. J Chromatogr 110:81–89
3. Smith DA, Cole WJ (1976) Identification of an arylesterase as the enzyme hydrolysing diacetylmorphine (heroin) in human plasma. Biochem Pharmacol 25:367–370
4. Lockridge O, Mottershaw-Jackson N, Eckerson HW, Ladu BN (1980) Hydrolysis of diacetylmorphine (heroin) by human serum cholinesterase. J Pharmacol Exp Ther 215:1–8
5. Boerner U, Abbott S, Roe RL (1975) The metabolism of morphine and heroin in man. Drug Metab Rev 4:39–73
6. Brunk SF, Delle M (1974) Morphine metabolism in man. Clin Pharmacol Ther 16:51–57

7. Shimomura K, Kamata O, Ueki S, Ida S, Oguri K, Yoshimura H, Tsukamoto H (1971) The analgesic effects of morphine glucuronides. Tohoku J Exp Med 105:45–52
8. Osborne R, Joel S, Trew D, Slevin M (1988) Analgesic activity of morphine-6-glucuronide. Lancet 1:828
9. Osborne R, Joel S, Trew D, Slevin M (1990) Morphine and metabolite behavior after different routes of morphine administration: demonstration of the importance of the active metabolite morphine-6-glucuronide. Clin Pharmacol Ther 47:12–19
10. Osborne R, Thompson P, Joel S, Trew D, Patel N, Slevine M (1992) The analgesic activity of morphine-6-glucuronide. Br J Pharmacol 34:130–138
11. Carrupt P-A, Testa B, Bechalany A, El Tayar N, Descas P, Perrissound D (1991) Morphine 6-glucuronide and morphine 3-glucuronide as molecular chameleons with unexpected lipophilicity. J Med Chem 34:1272–1275
12. Milne R, Nation R, Reynolds G, Somogyi A, van Crugten J (1991) High performance liquid chromatographic determination of morphine and its 3- and 6-glucuronide metabolites: improvements to the methods and application to stability studies. J Chromatogr 565:457–464
13. Wielbo D, Bhat R, Chari G, Vidyasagar D (1993) High-performance liquid chromatographic determination of morphine and its metabolites in plasma using diode-array detection. J Chromatogr 615:164–168
14. Steentoft A, Worm K, Pedersen C B, Sprehn M, Mogensen T, Sorensen MB, Nielsen E (1996) Drugs in blood samples from unconscious drug addicts after the intake of an overdose. Int J Legal Med 108:248–251
15. Bouquillon A, Freeman D, Moulin D (1992) Simultaneous solid-phase extraction and chromatographic analysis of morphine and hydromorphone in plasma by high-performance liquid chromatography with electrochemical detection. J Chromatogr 577:354–357
16. Mason J, Ashmore S, Aitkenhead A (1991) Simple method for the determination of morphine and its active glucuronide metabolite in human plasma by high-performance liquid chromatography with electrochemical detection. J Chromatogr 570:191–197
17. Verwey-van Wissen C, Koopman-Kimenay P (1991) Direct determination of codeine, norcodeine, morphine and normorphine with their corresponding O-glucuronide conjugates by high-performance liquid chromatography with electrochemical detection. J Chromatogr 570:309–320
18. Wright A, Watt J, Kennedy M, Cramond T, Smith M (1994) Quantitation of morphine, morphine-3-glucuronide and morphine-6-glucuronide in plasma and cerebrospinal fluid using solid-phase extraction and high-performance liquid chromatography with electrochemical detection. Ther Drug Monit 16: 200–208
19. Staub C, Jeanmonod R, Fryc O (1990) Morphine in postmortem blood: its importance for the diagnosis of deaths associated with opiate addiction. Int J Legal Med 104:39–42
20. Aderjan R, Hofmann S, Schmitt G, Skopp G (1995) Morphine and morphine glucuronides in serum of heroin consumers and heroin-related deaths determined by HPLC with native fluorescence detection. J Anal Toxicol 19:163–168
21. Joel S, Osborne R, Slevin M (1988) An improved method for the simultaneous determination of morphine and its principal glucuronide metabolites. J Chromatogr 430:394–399
22. Venn R, Michalkiewicz A (1990) Fast reliable assay for morphine and its metabolites using high-performance liquid chromatography and native fluorescence detection. J Chromatogr 525:379–388
23. Pawula M, Barrett D, Shaw N (1993) An improved extraction method for the HPLC determination of morphine and its metabolites in plasma. J Pharm Biomed Anal 11:401–406
24. Lemm U, Tenczer J, Baudisch H (1985) Antibody-mediated extraction of main tetrahydrocannabinol metabolite, 11-nor-Δ9- tetrahydrocannabinol-9-carboxylic acid, from human urine and its identification by gas chromatography-mass spectrometry in the sub-nanogram range. J Chromatogr Biomed Appl 342:393–398
25. Van de Water C, Haagsma N (1987) Determination of chloramphenicol in swine muscle tissue using a monoclonal antibody-mediated clean-up procedure. J Chromatogr 411:415–421
26. Van de Water C, Tebbal D, Haagsma N (1989) Monoclonal antibody-mediated clean-up for high-performance liquid chromatographic analysis of chloramphenicol in milk and eggs. J Chromatogr 478:205–215
27. Aderjan R, Schmitt G, Hofmann S (1994) Morphin und dessen Glucuronide im Serum von Heroinabhängigen. T + K 61, 2: 24–29