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ORIGINAL ARTICLE

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A specific immunoassay for the determination of morphine and its glucuronides in human blood

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Abstract The development of specific antisera for immunochemical determination of morphine, morphine-3-glucuronide and morphine-6-glucuronide is described. Morphine was N-demethylated to normorphine and N-alkylated to give N-aminopropyl-normorphine as hapten for antisera against morphine. As haptens for antisera against morphine-3-glucuronide and morphine-6-glucuronide, N-aminopropyl-nor-morphine was glucuronidated in position 3 or 6 respectively. Each of these three haptens were coupled to BSA employing the glutaraldehyde method to obtain three different immunogens. Immunisation of rabbits with these conjugates gave anti-morphine, anti-morphine-3-glucuronide and anti-morphine-6-glucuronide antisera, which were tested in a competitive, heterogeneous radioimmunoassay. Tracers for this radioimmunoassay procedure were synthesised by substitution of morphine and morphine-6-glucuronide in position 2 with ^{125}I and indirect iodination of the morphine-3-glucuronide hapten according to the method of Bolton and Hunter. The resulting antisera show very specific reactions with morphine, morphine-3-glucuronide and morphine-6-glucuronide. Cross reactivities of each antiserum with structurally related opiates and opioides are very low. The cross reactivities of the anti-morphine antiserum against morphine-3-glucuronide, morphine-6-glucuronide, codeine, codeine-6-glucuronide or dihydrocodeine were less than 0.3%, the anti-morphine-3-glucuronide antiserum against morphine, morphine-6-glucuronide, codeine, codeine-6-glucuronide or dihydrocodeine less than 0.1% and the anti-morphine-6-glucuronide antiserum against morphine, morphine-3-glucuronide, codeine or dihydrocodeine less than 0.1%, against codeine-6-glucuronide less than 2.3%. The determination of morphine, morphine-3-glucuronide and morphine-6-glucuronide in blood samples (limit of detection = 3, 1, 0.5 ng/g) of nine cases of fatal heroin overdose with this radioimmunoassay method and the comparison with a GC/MS method is described.

Key words Morphine · Morphine-3-glucuronide Morphine-6-glucuronide · Radioimmunoassay · RIA GC/MS

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Introduction

After the intake of 3,6-diacetyl morphine (heroin) a rapid deacetylation to 6-monoacetyl morphine and more slowly to morphine (M) follows [1–4]. M is then conjugated to glucuronic acid to form morphine-3-glucuronide (M3G) and to a lesser extent morphine-6-glucuronide (M6G). Furthermore morphine-3-ethereal-sulfate and normorphine are formed [5, 6]. The conjugation to glucuronic acid in the liver and intestine results in approximately 50% M3G which is pharmacologically inactive and an opiate antagonist and about 10% M6G which is considered to be a pharmacologically active metabolite with analgesic potency [7–11]. For these reasons the specific determination of M and morphine glucuronides is necessary in casework.

For the determination of drugs in a low concentration range immunoassay, especially radioimmunoassay (RIA) is one of the methods of choice because of the simple pretreatment of samples and large sample-throughput capacity. The specificity of such methods depends on factors such as the site of drug conjugation to carrier protein.

M was the first non-steroid drug for which RIA was developed by Spector and Parker in 1970 using 3-O-carboxymethyl morphine as hapten [12]. Since then many antisera (AS) against opiates have been developed using different positions in the target opiate to couple to the carrier protein. These studies showed that a clear relationship exists between immunogen structure and specificity of AS.

Binding to position 3 of M using haptens such as 3-O-carboxymethylmorphine [12, 13] or morphine-3-hemisuccinate [14] elicit AS which are not able to discern structural changes in position 3 from M. Also conjugation of M to carrier protein in position 6 employing haptens such as morphine-6-hemisuccinate [13, 15] or oxymorphone-6-(O-carboxymethyl)oxime [15] lead to AS which are not able to clearly recognize differences in positions 3 and 6 of the target molecule. The same problems occurred when using 2(p-aminophenylazo)-morphine-BSA as immunogen [13] or coupling BSA through the carboxylic group of M3G [16].

The best recognition of changes in positions 3 and 6 of M could be achieved with AS raised by attachment of M to BSA via the piperidine ring nitrogen [17]. The immunogen used in this case was N-carboxypropyl-normorphine-BSA. These studies showed that for the production of AS which should be able to recognize structural changes in positions 3 and 6 of M, these positions have to be unchanged as the epitope in the hapten when coupling to the carrier protein.

The aim of this study was to produce specific AS against M, M3G and M6G and the development of a RIA for the specific determination of M, M3G and M6G in blood samples. The best way seemed to be by employing immunogens which are synthesised by conjugation of M, M3G and M6G via the piperidine ring nitrogen to BSA. Therefore haptens were prepared by N-demethylation and N-alkylation of M to give N-aminopropyl-normorphine. After glucuronidation in position 3 or 6 these three haptens (N-aminopropyl-normorphine, N-aminopropyl-normorphine-3-glucuronide and N-aminopropyl-normorphine-6-glucuronide) were attached to BSA by the glutaraldehyde method [18]. After immunisation of rabbits the resulting polyclonal AS were used in a competitive heterogeneous RIA with ^{125}I labelled tracers. The results from blood samples in nine cases of fatal heroin overdose, analysed with this RIA procedure are compared with the results of GC/MS analysis, obtained after determination of free M and total M after cleavage of conjugated M by β-glucuronidase/aryl sulfatase.

Materials and methods

Chemicals

Morphine-3-β-D-glucuronide, morphine-6-β-D-glucuronide, naltrexone hydrochloride, heroin hydrochloride solution, buprenorphine hydrochloride, fentanyl citrate solution, cocaine, benzoylecgonin hydrate, Δ^9-tetrahydrocannabinol solution, ethylmorphine, 3-bromopropylamine hydrobromide (BPA × HBr) and glutaraldehyde (GA) solution were purchased from Sigma (Deisenhofen, Germany), morphine hydrochloride trihydrate, codeine hydrate, cyanogen bromide, silica gel 60 and β-glucuronidase/aryl sulfatase were obtained from Merck (Darmstadt, Germany), activated charcoal, silver carbonate, Schiff's reagent for aldehydes, 1,1,1,3,3,3-hexafluoro-2-propanol, N-methyl-bis-trifluoroacetamide (MBTFA) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) were purchased from Fluka (Neu-Ulm, Germany), iodo-gen iodination reagent and imject bovine serum albumin (BSA) from Pierce (Rockford, Il., USA), Na^{125}I 37 MBq and Bolton + Hunter reagent for protein iodination 18 MBq from Amersham Buchler (Braunschweig, Germany), codeine-6-β-D-glucuronide, 6-acetyl-morphine and normorphine from Lipomed (Arlesheim, Switzerland), pentafluoropropionic anhydride from Aldrich (Steinheim, Germany), bakerbond spe octadecyl 500 mg columns from Mallinckrodt Baker (Griesheim, Germany), worldwide monitoring clean screen dau, 500 and 200 mg columns from Restek (Sulzbach, Germany), sephadex G-25 pre-packed columns PD 10 from Pharmacia Biotech (Uppsala, Sweden), dihydrocodeine hydrogen tartrate from Knoll AG (Ludwigshafen, Germany), acetobromo-β-D-glucuronic acid methyl ester (ABGM) from Alkamed SARL (Bischheim, France), R-methadone hydrochloride from Hoechst (Frankfurt, Germany).

Instrumentation and chromatographic parameters

HPLC: a Hewlett Packard HP 1100 Series liquid chromatograph, consisting of binary pump (flow rate 1 ml/min), autosampler, column thermostat (40 °C) and diode-array detector (integration at 220 nm, spectra from 200 to 300 nm) were used. Chromatographic separation was performed with a LiChrospher 60 RP-select B (5 µm, 250 × 4 mm) analytical column (Merck, Darmstadt, Germany) using 10 mM KH_2PO_4 solution as mobile phase.

GC/MS: a Hewlett Packard 5890 Series II GC equipped with a 6890 Series autosampler and a 5972 series mass selective detector was used. For chromatographic separation a 5% phenyl-methyl silicone column HP-5MS (30 m × 0.25 mm i.d., 0.10 µm film thickness, Hewlett Packard) was used. Instrument parameters were as follows: injection volume 2 µl; injector temperature 250 °C; oven temperature programming for identification of synthesis products: initial temperature 150 °C held for 2 min, 20 °C/min to 300 °C held for 6 min; oven temperature programming for quantitative determination of free and total M: initial temperature 150 °C held for 1.5 min, 7 °C/min to 220 °C held for 8 min, 35 °C/min to 280 °C held for 5 min; interface temperature 280 °C; carrier gas helium; flow rate 1.0 ml/min. For identification of synthesis products total ions current was scanned in the EI mode in the range m/z 50 – m/z 700. For quantitative determination of free and total M selected ions were monitored in the EI mode at m/z 414, 577, and 357 for morphine pentafluoropropionyl derivative and m/z 459 and 296 for ethylmorphine pentafluoropropionyl derivative.

AP-ESI/MS: a Finnigan MAT LCQ instrument with the following parameters was used: spray voltage 2.59 kV, auxiliary gas flow rate 19.23 ml/min, capillary voltage 14.78 V, capillary temperature 250.4 °C, lens voltage –13.68 V, tube lens offset 25.0 V.

Buffer solutions

Phosphate buffer solution 0.1 M, pH 7.0 (coupling haptens to BSA): 17.8 g $Na_2HPO_4 \times 2\ H_2O$/l H_2O adjusted to pH 7.0 by addition of a solution of 13.6 g KH_2PO_4/l H_2O. Borate buffer 0.1 M, pH 8.6 (iodination of M3G tracer) consisted of 6.18 g H_3BO_3, 29 ml 1 M NaOH and 970 ml H_2O. Phosphate buffer saline (PBS, pH 7.4) contained 8.0 g NaCl, 1.4 g $Na_2HPO_4 \times 2\ H_2O$, 0.2 g KH_2PO_4, 0.2 g KCl and 0.2 g NaN_3/l H_2O. Acetate buffer solution 1 M, pH 5.5 (GC/MS determination of free and total M): 68.0 g natrium acetate trihydrate in 495 ml H_2O adjusted to pH 5.5 by addition of acetic acid (5 ml). Phosphate buffer solution 0.15 M, pH 6.0 (clean screen): 444.5 ml of a solution of 9.1 g KH_2PO_4/l H_2O was adjusted to pH 6.0 by addition of 55.5 ml of a solution of 11.9 g $Na_2HPO_4 \times 2\ H_2O$/l H_2O.

Synthesis of haptens

Normorphine was prepared from M by a slight modification of the methods of Von Braun [19] and Rapoport and Look [20]: 16.00 g (42.6 mmol) M HCl × 3 H_2O were dissolved in 75 ml acetic anhydride and the solution was refluxed for 5 h. Acetic acid and acetic anhydride were evaporated in vacuum at 10^4 Pa and 80 °C, the

glassy residual was dissolved in 20 ml H_2O, decolourized with activated charcoal and filtered, and when the pH was adjusted to 8–9 by addition of saturated Na_2CO_3 solution, heroin precipitated as a white precipitate. Drying at 105 °C resulted in a residue of pure heroin base. Of the heroin base 15.17 g (41.1 mmol) was dissoved in 75 ml $CHCl_3$ and a cold solution of 6.53 g (61.6 mmol) BrCN in 90 ml $CHCl_3$ was added dropwise under stirring within 30 min. The solution was stirred for 1 h at room temperature and then refluxed for 3 h. Evaporation of volatile material under reduced pressure gave a residue of cyanoheroin which was suspended in 60 ml 12 M HCl, refluxed for 5 min and after addition of 350 ml H_2O refluxed for another 8 h. The solution was filtered, concentrated in vacuum to ca. 200 ml and the pH was adjusted to 9.2 with ca. 120 ml 5 M NaOH. Precipitated normorphine was aspirated, washed with water, dried at 105 °C and yielded 7.24 g (26.7 mmol, 62.7% based on M HCl × 3 H_2O) pure normorphine.

N-(3-Trifluoracetamidopropyl)-normorphine (N-TFAAP-nor) was prepared by N-alkylation of normorphine with trifluoracetamidopropylbromide. For protection of primary amino function 11.64 g (53.1 mmol) BPA × HBr and 7.55 ml (53.1 mmol) MBTFA were dissolved in 50 ml dimethylformamide and stirred for 18 h under Ar at room temperature. After additon of another 2.85 g (13 mmol) BPA × HBr the solution was saturated with $NaHCO_3$ (pH 7–8) and added dropwise (within 90 min) to a solution of 7.24 g normorphine base in 75 ml dimethylformamide which was heated to 70 °C. The reaction mixture was stirred for 6 h at 70 °C, adjusted to pH 9.2 with saturated Na_2CO_3 solution and extracted four times with each 75 ml ethyl acetate. The collected organic phases were dried over Na_2SO_4, decolourized with activated charcoal and evaporated at 2×10^4 Pa and 40 °C. The residue was chromatographed on a column of 50 g silica gel 60 with ethyl acetate/methanol (85/15 by vol.) as mobile phase. The first 80 ml mobile phase were discarded and from 80 to 250 ml three fractions were collected and identified by GC/MS after silylation as trimethylsilyl derivatives. Evaporation of solvent gave 5.52 g (13.0 mmol, 48.8% based on normorphine) of a pale yellow viscous mass of N-(3-trifluoracetamidopropyl)-normorphine.

Identification: GC/MS, EI: N-TFAAP-nor × 2 TMS $m^+ = 568$
AP-ESI/MS: $m^{+1} = 425.2$

N-(3-Aminopropyl)-normorphine (N-AP-nor) was synthesised by alkaline hydrolysis of N-TFAAP-nor and 60 mg (141 µmol) N-TFAAP-nor was dissolved in 600 µl methanol and 1.89 ml 0.75 M NaOH in methanol (1.41 mmol NaOH) was added. The solution was heated up to 70 °C under Ar for 2 h, neutralized by addition of 900 µl acetic acid and evaporated under a stream of nitrogen at 40 °C. The residual was dissolved in phosphate buffer pH 6.0 and purification was carried out with clean screen 500 mg solid phase extraction columns (see manufacturers instructions). Evaporation of solvent under a stream of nitrogen at 40 °C resulted in 32 mg (97 µmol, 68.8% based on N-TFAAP-nor) of pale yellow amorph N-AP-nor.

Identification: HPLC/DAD: typical spectrum of opiates
AP-ESI/MS: $m^{+1} = 329.3$
CG/MS, EI: N-AP-nor × 4 TMS $m^+ = 616$

N-(3-Aminopropyl)-normorphine-3-β-D-glucuronide (N-AP-nor-3-g) was prepared by glucuronidation of N-TFAAP-nor with ABGM. To a solution of 3.17 g (7.47 mmol) N-TFAAP-nor in 50 ml methanol, 0.34 g of LiOH × $2H_2O$ and 4.73 g ABGM (11.91 mmol) were added. After stirring for 3 h at room temperature 0.34 g LiOH × 2 H_2O and 50 ml water were added. After another 15 h at room temperature the solvents were evaporated. The residue was dissolved in 50 ml water, the reactio mixture was adjusted to pH 9 with saturated Na_2CO_3 solution and extracted four times with methylene chloride/methanol (3/1 by vol.) to remove unreacted morphine. The aqueous layer was concentrated under vacuum and crude N-AP-nor-3-g was precipitated by additon of ethanol. After separation by centrifugation, followed by drying at 105 °C, this product was recrystallised from ethanol/water (97.5/2.5 by vol, 7 ml/g of product) and dissolved in water. Purification was carried out by column chromatography on 50 g octadecyl silice. The column was washed with water and pure N-AP-nor-3-g was collected by gradient elution with water/methanol (10/1–1/1 by vol.) in 25 ml fractions. These fractions were identified by HPLC/DAD and concentrated under reduced pressure. Precipitation with ethanol, separation by centrifugation and drying at 105 °C gave 0.45 g (0.89 mmol, 11.9%) of pale yellow N-AP-nor-3-g.

Identification: HPLC/DAD: typical spectrum of opiates
AP-ESI/MS: $m^{+1} = 505.2$

N-(3-Aminopropyl)-normorphine-6-β-D-glucuronide (N-AP-nor-6-g) was prepared by glucuronidation of 3-acetyl-N-TFAAP-nor with ABGM. To 5.20 g N-TFAAP-nor (12.26 mmol) 250 ml 1 M $NaHCO_3$ solution and 12.5 ml Ac_2O were added in five portions each of 50 ml 1 M $NaHCO_3$ solution and 2.5 ml Ac_2O within 24 h under stirring of the mixture at room temperature. Adjusting pH to 8.5 with saturated $NaHCO_3$ solution and $NaCO_3$ solution led to a pale yellow precipitate which was extracted four times with CH_2Cl_2 and evaporated to dryness. The oily yellow residue (3.11 g, ~6.7 mmol, mixture of 3-acetyl-N-TFAAP-nor and 3,6-diacetyl-N-TFAAP-nor in the ratio 2 to 1, detected by GC/MS) was dissolved in 250 ml toluene and 6.44 g Ag_2CO_3 (23.36 mmol) and 10.59 g ABGM (26.68 mmol) were added. The mixture was refluxed for 6 h, 4.76 g ABGM was added and refluxing was continued for 10 h. Ag_2CO_3 was separated by filtration and the organic phase was concentrated by evaporation under reduced pressure. Under stirring at room temperature and permanent control of pH 400 ml 0.1 M NaOH solution and 250 ml methanol were added in four portions within 24 h until a constant pH of about 9 was reached. After neutralisation with acetic acid and concentration to 50 ml under reduced pressure the crude N-AP-nor-6-g was precipitated with EtOH, separated by centrifugation and dissolved in 100 ml H_2O. This solution was cleaned up by solid phase extraction with octadecyl silice 500 mg columns. Each 5 ml of the crude solution was applied to a column (all in all 20 columns were used) pretreated with methanol and water. The columns were washed with four portions of 2.5 ml water. Elution of N-AP-nor-6-g was carried out by gradient elution with six portions of 1 ml water containing 10% methanol to 60% methanol. The collected fractions were analysed by HPLC/DAD, the N-AP-nor-6-g containing fractions 3, 4 and 5 were mixed and pure N-AP-nor-6-g was precipitated by addition of EtOH. The pale yellow precipitate (74 mg, 147 µmol, 1.2% based on N-TFAAP-nor) was separated by centrifugation and dried at 105 °C.

Identification: HPLC/DAD: typical spectrum of opiates
AP-ESI/MS: $m^{+1} = 505.1$

Preparation of immunogens

For the preparation of immunogens, the carrier protein BSA was activated by introduction of aldehyde functions with glutaraldehyde and then haptens were coupled to this activated BSA. To calculate the ratio of moles hapten per mol carrier protein the amount of aldehyde groups in activated BSA before coupling to haptens and after coupling was determined spectrophotometrically [21].

Activation of BSA

To a solution of 150 mg BSA (2.24 µmol) in 10 ml phosphate buffer solution 0.1 M pH 7.0, a solution of 180 mg 25% GA solution in 5 ml phosphate buffer (45 mg GA, 449.55 µmol GA) was slowly added (BSA: GA = 1 mol: 200 mol). During stirring at room temperature for 90 min the solution turned yellow. To quickly remove the remaining GA the reaction mixture was immediately transferred to pre-packed sephadex G-25 columns for gel filtration (see manufactures instruction). After elution of the BSA-GA conjugate the volume of the solution increased to 18 ml. The activated BSA was immediately used for coupling to the haptens.

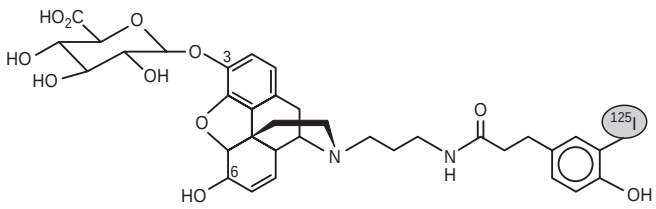


Fig. 1 Molecular structure of the radio tracer for the determination of M3G, synthesised by indirect iodination of N-(3-Aminopropyl)-normorphine-3-β-D-glucuronide

Coupling haptens to BSA

N-AP-nor-BSA: a solution of 26.3 mg N-AP-nor (80.2 μmol) in 2.5 ml buffer solution was added dropwise to 6 ml activated BSA solution (50 mg BSA, 0.75 μmol) and after addition of 1.5 ml buffer solution the mixture was stirred overnight at room temperature. Gel filtration and lyophilisation of the elutate led to the pure immunogen. For N-AP-nor-3-g-BSA 25.5 mg N-AP-nor-3-g (50.6 μmol) was coupled to 33.3 mg activated BSA (0.50 μmol) and for N-AP-nor-6-g-BSA 15.0 mg N-AP-nor-6-g (29.8 μmol) was bound to 20 mg BSA (0.30 μmol).

Immunisation and collecting antisera

The immunisation of rabbits (Bauer, Neuenstein) was carried out at the Behring-Diagnostics GmbH Marburg by subcutaneous and intravenous injections. The immunogens were dissolved in isotonic NaCl solution and mixed with Complete Freund's Adjuvant for subcutaneous injection and Aerosil for intravenous injections. Blood samples were taken 38, 52 66, 80 88 101 and 115 days after priming and at day 122 the rabbits were sacrificed and the whole blood was collected.

Synthesis of tracers

For the application of the antisera in a radioimmunoassay, M, M3G and M6G were radiolabelled with ^{125}I. For the preparation of M-tracer and M6G-tracer Na^{125}I was oxidised by Iodo-gen (1,3,4,6-tetrachloro3,6-diphenylglycouril) and M and M6G were iodinated in position 2 by electrophil substitution with ^{125}I as described by Chapman et al. [22, 23].

The M3G-tracer was synthesised by indirect iodination of N-AP-nor-3-g by the method of Bolton and Hunter [24, 25] (see Fig. 1). The Bolton+Hunter reagent containing N-succinimidyl 3-(4-hydroxy,5-[^{125}I]iodophenyl)-propionate in benzene was evaporated with dried He as described in the manufacturers instructions. A solutionof 25.2 μg N-AP-nor-3-g (50 nmol) in 50 μl borate buffer solution 0.1 M pH 8.6 was added, mixed and after 15 min transferred to a conditioned C18 SPE-column (500 mg). The column was washed with eight portions of 2 ml water and the ^{125}I labelled N-AP-nor-3-g was eluted in five portions of 2 ml methanol. The activity of these fractions were determined in a gamma counter and the fraction with the highest activity (No. 1) was used as tracer for determination of M3G.

Radioimmunoassay

A heterogeneous competitive radio-immunoassay was chosen for testing antisera and for the determination of M, M3G and M6G in blood samples from heroin-related deaths. The tracer solutions were diluted with PBS to a final activity of ~30.000 CPM. For the determination of antisera titer the antisera were diluted from 1:100 to 1:10.000 with PBS, 100 μl diluted antiserum was mixed with 100 μl tracer solution and incubated for 1 h at room temperature. The antibody-antigen complex was precipitated by addition of 200 μl saturated $(NH_4)_2SO_4$ solution, separated by centrifugation at 3000 g for 30 min and after aspiration of the supernatant the activity of the remaining pellet was counted for 1 min in the ^{125}I canal of a gamma counter.

To determine the specificity of the antisera (50% displacement method [26]). 100 μl solutions of potent cross reactants in concentrations ranging from 0 to 10,000 ng/ml were mixed with 100 μl tracer solution and 100 μl of diluted antiserum. Precipitation was carried out with 300 μl saturated $(NH_4)_2SO_4$ solution.

For the determination of M, M3G and M6G in blood samples the radioimmunoassay was calibrated with spiked blood samples in the concentration range 1 ng to 1000 ng analyte/g blood. For precipitation of proteins 0.5 ml methanol was added to 0.5 g whole blood (spiked standards and samples from heroin victims). The mixture was diluted with 1 ml PBS, treated with ultra sonic for 5 min and centrifuged at 3000 g for 5 min. Of the supernatant 200 μl was used for the RIA procedure, mixed with 100 μl tracer solution and 100 μl diluted antiserum and incubated for 1 h at room temperature. Precipitation was carried out by addition of 400 μl saturated $(NH_4)_2SO_4$ solution and after centrifugation for 30 min at 3000 g and aspiration of the supernatant the pellet was counted for 1 min.

GC/MS

The GC/MS analysis of free and total M was executed by solid phase extraction and derivatization [27] of morphine before and after cleavage of conjugates. For calibration of GC/MS analysis of free M, calibration standards were prepared by spiking blood in the concentration range 50–500 ng M/g. For the determination of total M, blood was spiked in the concentration range 50–1000 ng M3G/g. Calibration standards and blood samples of heroin victims were treated in the same way.

To 2 g blood (calibration standards and samples of heroin victims) 100 ng ethylmorphine (100 μl of a solution of 1 μg/ml in methanol) as internal standard and 2 ml acetate buffer (pH 5.5) were added and the samples were mixed.

For conjugate cleavage 200 μl β-glucuronidase/aryl sulfatase was added to samples and after mixing incubated for 12 h at 38 °C. Samples without conjugate cleavage were kept cool.

After addition of 2 ml methanol the samples were mixed intensively and treated with ultra sonic for 5 min, 10 ml phosphate buffer pH 6.0 was added and after mixing treatment with ultra sonic for 5 min and centrifugation at 3000 g for 10 min the supernatants were applied to a pretreated column for solid phase extraction. The extraction was carried out with clean screen SPE columns (see manufacturers instruction). The eluates were evaporated under a stream of nitrogen at 50 °C. For derivatisation as pentafluoropropionyl derivates, 50 μl pentafluoropropionic anhydride and 50 μl hexafluoroisopropanol were added to the dried extracts, the samples were mixed, kept at 70 °C for 30 min, again evaporated under a stream of nitrogen at 50 °C, dissolved in 50 μl ethyl acetate and injected into GC/MS.

Results and discussion

For the specific determination of M, M3G and M6G in whole blood by RIA, three different immunogens were synthesised which led to three different specific antisera against M, M3G and M6G after the immunisation of rabbits.

During the period of immunisation several blood samples were taken. To control the production and development of specific antisera the ability for binding radiolabelled tracers was determined (Fig. 2). Dilutions of anti-

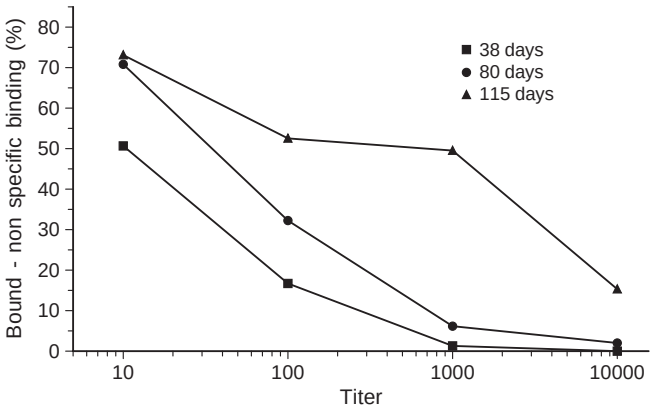


Fig. 2 Development of an antiserum against M3G, expressed as the ability of binding a radio tracer in relation to dilution of antiserum, 38, 80 and 115 days after priming of the rabbit

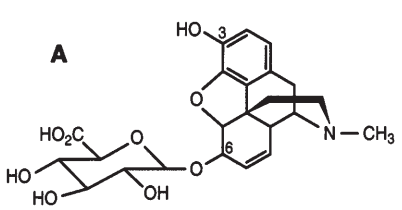
sera in the range 1:10 to 1:10,000 were incubated with a constant quantity of tracer. After separation of the precipitated antibody-antigen complex the amount of bound tracer was determined. After subtraction of the non-specific binding the amount of bound tracer was calculated as a percentage of the origin tracer amount. After 115 days the quality of antisera did not increase and 7 days later the rabbits were sacrificed to collect whole blood. For the determination of cross reactivity and the concentration of M, M3G and M6G in blood samples of heroin victims, the anti-M antiserum was used in the dilution of 1:500; the anti-M3G antiserum in the dilution 1:1500 and the anti-M6G antiserum in the dilution 1:1000 in a heterogeneous competitive RIA.

The specificity of the resulting antisera were screened by the measurement of cross reactivity employing the 50% displacement method. Therefore the analytes were employed in the concentration range 1–1000 ng/ml, the cross reactants in the range 1 ng–100 μg/ml. Thus the range to be expected in real samples is covered. As potential cross reactants structurally related opiates and opioides were chosen. In addition to these substances typical drugs of abuse were tested. In Fig. 3 the target substance M6G and the synthesised immunogen for the development of antisera against this target substance is presented. This example shows that the structure in position 3 and 6 of the target substances could be preserved in the structure of immunogens because the coupling to carrier protein was carried out by reaction of the aldehyde groups of glutaraldehyde with the amino groups of haptens and ε-amino groups of lysin in BSA. This structure of immunogens enabled the presentation of the positions 3 and 6 as antigenic determinants and immunisation of animals led to antisera which are specific in recognition of changes in these positions. In Table 1 the results of the determination of cross reactivity are given. Potential cross reactants with

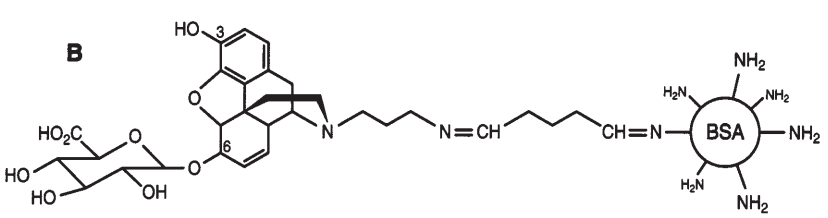
Table 1 Cross reactivity (%) of various opiates, opioides and other typical drugs of abuse. Morphine, M3G and M6G as analytes were employed in the concentration range 1–1000 ng/ml as cross reactants and the other cross reactants in the range 1 ng–100 μg/ml

	Aniserum against		
	Morphine	M3G	M6G
Morphine	100	< 0.02	< 0.01
M3G	0.27	100	< 0.01
M6G	0.19	0.06	100
Codeine	0.13	< 0.02	< 0.01
Codeine-6-glucuronide	< 0.01	< 0.02	2.25
Dihydrocodeine	0.09	< 0.02	< 0.01
Heroin	2.59	< 0.02	< 0.01
6-acetyl-morphine	1.00	< 0.02	< 0.01
Normorphine	6.82	< 0.02	0.29
Naltexone	0.10	< 0.02	< 0.01
Buprenorphine	< 0.01	< 0.02	< 0.01
Fentanyl	< 0.01	< 0.02	< 0.01
R-methadone	0.09	< 0.02	< 0.01
Cocaine	< 0.01	< 0.02	< 0.01
Tetrahydrocannabinol	< 0.01	< 0.02	< 0.01
Amphetamine	< 0.01	< 0.02	< 0.01
Diazepam	< 0.01	< 0.02	< 0.01

Fig. 3 Structural relationship between target molecule (A) and immunogen (B) shown at the example of M6G



Morphine-6-β-D-glucuronide



Immunogen for antisera against morphine-6-β-D-glucuronide

Table 2 The concentrations for free and bound morphine (determined by GC/MS) and for morphine and morphine glucuronides (determined by RIA) in nine cases of heroin-related death

Case no.	GC/MS (ng/g blood)			RIA (ng/g blood)	
	Free morphine	Bound morphine	Morphine	M3G	M6G
1	272	188	250	250	49
2	164	213	180	297	35
3	450	319	395	406	64
4	303	490	246	581	123
5	132	96	110	110	25
6	139	202	152	270	47
7	317	369	285	529	70
8	25	46	22	70	8
9	95	310	92	400	68

structural changes in position 3 or 6 in relation to the target substance always have negligible cross reactivity. So M-metabolites have no influence on the determination of M, M and M6G do not disturb the detection of M3G and M and M3G do not affect the detection of M6G.

The calibration of the RIA was carried out with calibration standards (spiked blood samples) in the concentration range 1–1000 ng/g. So samples of heroin victims and calibration standards were handled in the same way. The addition of 1 ng analyte/g blood (M, M3G or M6G) as a calibration standard leads to a strong displacement of tracer from antibody binding sites and to a significant reduction of signal. So the limit of detection (blank - 3σ) for this test system is 3 ng/g blood for M, 1 ng/g for M3G and 0.5 ng/g for M6G.

To control the results of this RIA procedure the blood samples of nine heroin victims were examined by RIA and GC/MS. The results of these determinations are presented in Table 2. In cases1–7 there was an acute intoxication with an obvious overdose of heroin. The circumstances of discovery of the bodies and the autopsy findings confirmed this. In case 8, 959 ng methadone/g blood were found, in case 9, a witness reported that the victim survived for 9 h after the last injection of heroin.

The findings for free morphine from RIA and GC/MS could be compared directly and the correlation of these determinations are shown in Fig. 4A. There is a sufficient correlation between the results of both methods. For the comparison of morphine metabolites bound M per GC/MS was calculated by subtracting free M from total M after cleavage of conjugates by β-glucuronidase/aryl sulfatase. The RIA findings for M3G and M6G were added and calculated as M. Also this correlation (Fig. 4B) is very good. In seven of the nine cases, the results for bound M per GC/MS were higher than the sum of M3G and M6G, calculated as M. The reason for this may be that β-glucuronidase/aryl sulfatase also cleaves morphine sulfate so that this occurs in the findings for bound morphine. The data from the RIA do not include morphine sulfate.

The presented RIA test is a very sensitive and rapid quantitative method for the determination of M, M3G and M6G in blood samples. The main advantage of this procedure is the low limit of detection and its ability to differentiate between pharmacologically inactive and analgesic potent metabolites of heroin.

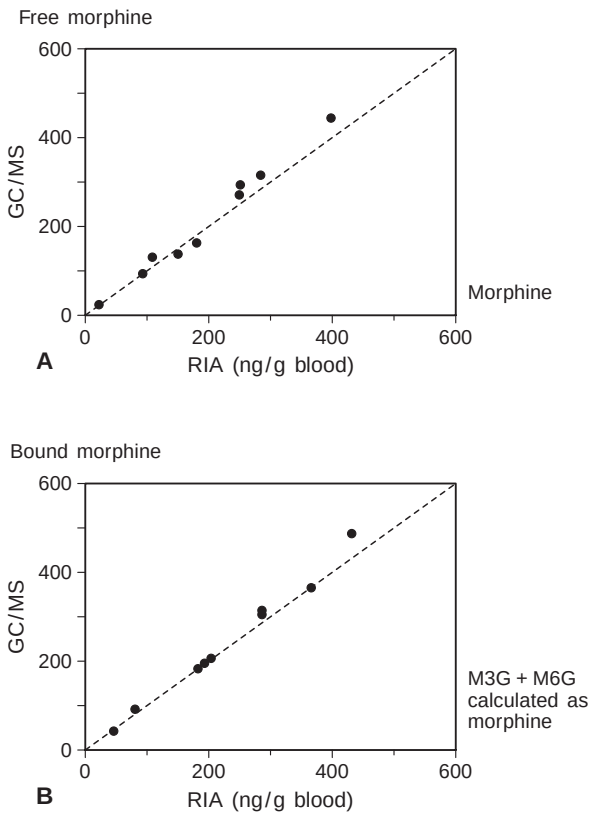


Fig. 4 A, B Correlation between GC/MS and RIA in 9 cases of heroin-related death. **A**: Comparison of the results of GC/MS determination of free morphine with the results of RIA determination of morphine. **B**: Comparison of GC/MS results for bound morphine (difference between total morphine and free morphine) with the sum of M3G and M6G, calculated as morphine

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