Original Article

Endogenous Morphine-6-Glucuronide (M6G) is Present in the Plasma of Patients: Validation of a Specific Anti-M6G Antibody for Clinical and Basic Research

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Abstract

Endogenous morphine and its derivatives [morphine-6-glucuronide (M6G); morphine-3-glucuronide (M3G)] are formed by mammalian cells from dopamine. Changes in the concentrations of endogenous morphine have been demonstrated in several pathologies [sepsis, Parkinson's disease, etc.], and they might be relevant as pathological markers. While endogenous morphine levels are detectable using enzyme-linked immunosorbent assay (ELISA), mass spectrometry analysis was, so far, the only approach to detect and quantify M6G. This study describes the preparation of a specific anti-M6G rabbit polyclonal antibody and its validation. The specificity of this antibody was assessed against 30 morphine-related compounds. Then, a M6G-specific ELISA-assay was tested to

quantify M6G in the plasma of healthy donors, morphine-treated, and critically ill patients. The antibody raised against M6G displays a strong affinity for M6G, codeine-6-glucuronide, and morphine-3-6-glucuronide, whereas only weak cross-reactivities were observed for the other compounds. Both M6G-ELISA and LC-MS/MS approaches revealed the absence of M6G in the plasma of healthy donors (controls, $n=8$). In all positive donors treated with morphine-patch ($n=5$), M6G was detected using both M6G-ELISA and LC-MS/MS analysis. Finally, in a study on critically ill patients with circulating endogenous morphine ($n=26$), LC-MS/MS analysis revealed that 73% of the positive-patients (19 of 26), corresponding to high M6G-levels in M6G-ELISA, contained M6G.

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Volume 000, Number 000, Month/Month 2013, Pages 000-000

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Received February 2013; accepted 3 April 2013

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Additional Supporting Information may be found in the online version of this article.

DOI: 10.1002/biof.1107

Published online 00 Month 2013 in Wiley Online Library (wileyonlinelibrary.com)

In conclusion, we show that endogenous M6G can be found at higher levels than morphine in the blood of morphine-naïve patients. With respect to the interest of measuring endogenous M6G in pathologies, we provide evidences that our

ELISA procedure represents a powerful tool as it can easily and specifically detect endogenous M6G levels. © 2013 BioFactors, 00(00)000-000, 2013

Keywords: morphine; morphine 6 glucuronide; ELISA; critically ill patient

1. Introduction

Morphine from *Papaver somniferum* remains the ultimate drug to treat pain. In humans, morphine is mainly metabolized into morphine-6-glucuronide (M6G) and morphine-3-glucuronide (M3G) through the action of the UDP-glucuronosyltransferase families 1A and 2B (UGT1A, UGT2B) present in the liver (1) and in the brain (2). M6G displays a greater analgesic effect than morphine (3), whereas M3G is, without effect on opioid receptors (4). While morphine and M6G bind the mu opioid receptor (MOR), thus inducing analgesia, M3G and morphine also interact with the inflammation-transducing receptor TLR4 (toll-like receptor 4), an action that may explain its proalgesic and proinflammatory effects (4). These exciting data suggest that the activation of MOR and TLR4 is also dependant of the morphine/M3G/M6G ratios and is crucial for the modulation/plasticity of physiological and pathological processes.

Similarly, to what is seen in plants, the synthesis of endogenous morphine (eM), M6G, and M3G occurs in different mammalian tissues and cells (3,5-8). A recurrent concern deals with morphine of vegetal origin contained in the food. Indeed, morphine has been detected in plants including hay and lettuce (2-10 ng/g of dry weight), as well as in cow's and human breast milk (at the concentrations ranging from 200 to 500 ng/L) (9). These findings point out a potential morphine contamination coming from diet. However, experiments carried on in strict morphine-free conditions, with radioactive elements as precursors and in tissue cultures devoided of any trace of morphine, confirmed that *de novo* eM synthesis is occurring in mammals (10).

We have previously reported the presence of M6G in secretory granules and secreted material of bovine adrenal chromaffin cells (11). M6G was found to be associated with the phosphatidyl-ethanolamine-binding protein in these organelles. In these particular cells, we have determined the presence of an UGT2B-like enzyme able to convert morphine into M6G. More recently, using the human SH-SY5Y cell line model, we observed the presence of both morphine and M6G and characterized the presence of the UGT2B7 (12).

Several studies report an increase in eM amounts in blood, urine or tissue collected from various pathological conditions, including infection, inflammation (13-18), or neurologic disease such as bulimia, anorexia and Parkinson disease (19,20). More precisely, eM concentrations reach an average concen-

tration of 2.5 ng/mL in serum of patients suffering from systemic infections (sepsis). Healthy donors and control patients have nondetectable or very low eM concentrations in the blood (16,18). For these reasons, quantification of new endogenous morphine-derived compounds, such as M6G, could represent a useful pathological marker.

Presence and increases in endogenous M6G have been rarely studied due to the lack of quantification tools outside LC-MS/MS analysis. Only few enzyme-linked immunosorbent assay (ELISA) assays against M6G have been previously proposed (21). As endogenous M6G is present at low concentrations in biological fluids, the quantification method to be implemented has to be highly sensitive. In addition, the presence of other alkaloids, closely-related in their structure (i.e., M3G, morphine, and codeine), requires a highly specific quantification method.

In this article, we characterize a highly specific anti-M6G rabbit polyclonal antibody. This antibody was validated for ELISA and its cross-reactivity was investigated using 30 compounds structurally-related to M6G. Finally, a M6G-specific ELISA-prototype was produced and used in parallel of a LC-MS/MS analysis to determine M6G levels in the plasma of controls, morphine treated patients and critically ill patients.

2. Materials and Methods

2.1. Materials and Chemicals

Keyhole limpet hemocyanin (KLH), glutaraldehyde, triethanolamine (TEA), bovine serum albumin (BSA) protease free, avidin, and dimethylformamide anhydrous (DMF) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium borohydride (NaBH₄) was from Acros (Geel, Belgium). Biotin-NHS was from Calbiochem (Darmstadt, Germany). H₂SO₄ was obtained from Merck (Darmstadt, Germany). StabilZyme HRP Conjugate Stabilizer was from Surmodics (Eden Prairie, MN). Chromogenic substrate TMB (3,3',5,5'-tetramethylbenzidine) was from KPL (Gaithersburg, MD). The 96-well microplates were from NUNC (Roskilde, Denmark). New-born calf serum (NBS) was from PAA laboratories (Linz, Austria). Activated horseradish-peroxidase (HRP) was from Roche diagnostics (Meylan, France). HiTrap Protein A HP Columns and Sephadex HR10/30 were from GE Healthcare Life Sciences (Chalfont St. Giles, UK). OASIS HLB and MC-X SPE-cartridges (1 cc, 30 mg) were from Waters

(Milford), SOLA, SPE-cartridges were from Thermo Fisher Scientific (Mlirsch, France). Bond Elute Certify SPE-cartridges were from Agilent (Massy, France). Strata-X 33u Polymeric Reversed Phase, Strata-XL 100u Polymeric Phase, Strata C18-E, Strata SAX, Strata Screen-A, Strata X-AW 33u Polymeric Weak Anion, Strata-X-CW 33u Polymeric Weak Cation, Strata WCX, Strata-X-C 33u Polymeric Strong Cation, Strata-SL-C 100u Polymeric Strong Cation, Strata Screen-C and Strata-X-C 33u Polymeric Strong Cation SPE-cartridges were from Phenomenex (Le Pecq, France). Chromabond Drug SPE-cartridges were from Macherey Nagel, Hoerd, France). Trap column Amide-80 (10mm length $\times$ 2mm inner diameter (I.D.), 5 μ m) and TSKgel Amide-80 (25cm length $\times$ 1mm I.D., 5 μ m) were from TOSOH (Tokyo, Japan). Dialysis tubing and AMICON ultra pore were from Millipore (Molsheim, France). Alkaloids and MOR agonists and antagonists were either from Sigma-Aldrich or Abcam Biochemicals (Cambridge, UK). LH-PSTII tubes (reference no. 367373) were from BD (Le Pont de Claix, France). Morphine-specific ELISA kits were from Immunalysis (reference no. 213-0480, Pomona). Other chemicals compounds were from Merck and Sigma-Aldrich.

2.2. Preparation of M6G-Immunogen

The M6G-3-NH₂ hapten (supplementary files) was coupled to KLH by glutaraldehyde condensation reaction using a molar ratio of 50:1 for hapten:protein. Hapten of 832 nmol was diluted in phosphate buffer saline (PBS; 0.9%NaCl and 25 mM sodium phosphate, pH 7.4) was added to 16.5 nmol of KLH leading to a mixture of 1.2 mL. Then 144 μ L of glutaraldehyde 10% (v/v) was added and the mixture was stirred 2 H at room temperature (RT). The reaction was stopped with NaBH₄. The resulting conjugate was then dialyzed against PBS over 24 H, aliquoted and stored at -20°C.

2.3. Immunization Protocol

The immunogen described above was diluted in PBS pH 7 (1 mg/mL), mixed with complete Freund's adjuvant and injected subcutaneously in two males New Zealand rabbits. A first boost was given at day 30 after the initial inoculation and subsequent boosts were given every 30 days. Blood were taken after the second boost from the marginal ear vein and after a one week intervals following each immunization. The anti-serum obtained was stored at -20°C.

The titer of the rabbit antiserum was monitored by ELISA. The anti-serum having the highest titer was then purified using HiTrap Protein A-HP columns according to manufacturer instructions.

2.4. Antibody, Standard, and Controls Preparation

The rabbit anti-M6G polyclonal antibody was biotinylated for the ELISA assay. After dialysis in buffer carbonate 100 mM pH 9, biotin-NHS dissolved in DMF was added to the antibody. After 30 Min of stirring, the free biotin was removed by dialysis in PBS 11 mM pH 7.2. Aliquots of labeled antibody were frozen and stored at -20°C. Then, the biotinylated antibody was

diluted (0.25 μ g/mL) in PBS 11 mM 1%-BSA (m.v, pH 7.2), aliquoted and stored at -20°C. Calibration curves were prepared in NBCS by spiking M6G over the range from 0 to 20 ng/mL. Internal controls with were prepared using NBCS for control (0 ng/mL M6G) and pools of pathological serum with low (1.26 ng/mL), medium (3.13 ng/mL) and high (6.34 ng/mL) M6G levels of M6G, aliquoted and stored at -20°C.

2.5. M6G Conjugate

The M6G-3-NH₂ hapten was coupled to HRP to synthesize M6G ELISA tracer. A molar ratio of 5:1 was used (hapten:protein). Conjugation was done according to the manufacturer instructions. Briefly, 27 μ g of M6G-3-NH₂ was added to 1 mg of activated HRP in sodium carbonate buffer 1 M pH 9.4. The mixture was stirred overnight at 4°C. To stop the reaction, 40 μ L of TEA 2 M pH8 and 50 μ L of NaBH₄ were added. The mixture was left stirred 30 Min at 4°C. A 25- μ L TEA was added again and the final mixture was incubated 2 H at 4°C. Finally, 10 μ L of glycine 1 M (pH 7) was added to stabilize the conjugate. Purification of the conjugate was done using gel filtration Superdex HR10/30. Finally, the conjugate was diluted 30 folds in Stabilzyme, aliquoted and stored at 4°C. For ELISA assays, the conjugate was diluted at working concentration (100 ng/mL) in Stabilzyme.

2.6. M6G-ELISA Microplates Preparation

96 wells microplates were coated with 200 μ L/wells of a 10- μ g/mL BSA-biotin solution in PBS (24 H at RT). Then after aspiration 200 μ L/wells of 10 μ g/mL of avidin solution in PBS was distributed and incubated 24 H at RT. After washing with 0.8% NaCl (m.v), 0.005% Tween20 (v.v), 200 μ L/wells of saturation solution was added (PBS, 2% Sorbitol (m.v), 0.5% Glycine (m.v), 0.1% BSA (m.v) and 0.065% NaN₃ (m.v)) and left incubated 24 H at 4°C. After aspiration, the microplates were dried at 20°C in a ventilated room for 12 H, packed with a desiccant and stored at 4°C.

2.7. M6G-ELISA Procedure

A competitive assay format was used for the M6G-ELISA. A 100- μ L biotinylated antibody was added and incubated for 2 H at RT (400 rpm stirring). After incubation, the wells were emptied and three wash cycles with H₂O supplemented with 0.05% Tween20 (v.v) were applied. Approximately of 50- μ L calibrators, controls (NBCS for control, 0 ng/mL M6G; pools of pathological serum with low, 1.26 ng/mL, medium 3.13 ng/mL and high 6.34 ng/mL M6G levels), or samples were added in duplicate. If necessary, samples were prediluted in dilution buffer (PBS + 0.1% BSA). Then, 50 μ L of M6G-HRP conjugate was distributed in all wells and incubated 2 H under agitation at RT. After a final washing step, 100 μ L of HRP substrate (TMB) was added, and left incubated 10 Min under agitation at RT. The chromogenic reaction was stopped with 100 μ L H₂SO₄ 1 N, and the absorbance of each sample was determined at 450nm and 620 nm.

Results were analyzed using GraphPad Prism Software. Patient samples were analyzed in duplicated in a blinded fashion. All samples with a duplicate CV > 5% were retested to obtain a CV below or equal to 5%.

2.8. Anti-M6G Antibody's Specificity

Determination of anti-M6G antibody specificity was performed using the M6G ELISA procedure as described above. An amount of 50 μ L of the following potential cross-reactants (0.01 nM to 1 μ M, in duplicate) were used to determine cross-reactivities:

2.8.1. Alkaloids

morphine [7,8-dihydro-4,5-epoxy-17-methylmorphinan-3,6-diol, CAS (57-27-2)], M6G [CAS (20290-10-2)], morphine-3- β -D-glucuronide [CAS (20290-09-9)], codeine [(5R,6S)-7,8-didehydro-4,5-epoxy-3-methoxy-N-methylmorphinan-6-ol, CAS (76-57-3)], codeine-6- β -D-glucuronide [CAS (20736-11-2)], morphine-3- β -D-glucuronide [CAS (64947-41-7)], norcodeine [3-methoxy-6 α -hydroxy-4,5 α -epoxy-7,8-didehydromorphinan, CAS (467-15-2)], normorphine [3,6 α -dihydroxy-4,5 α -epoxy-7,8-didehydromorphinan, CAS (466-97-7)], hydromorphone [4,5 α -epoxy-3-hydroxy-17-methylmorphinan-6-one, CAS (466-99-9)], hydrocodone [4,5 α -Epoxy-3-methoxy-17-methylmorphinan-6-one, CAS (125-29-1)], thebaine [6,7,8,14-tetrahydro-4,5 α -epoxy-3,6-dimethoxy-17-methylmorphinan, CAS (115-37-7)],

2.8.2. Monoamines and catecholamines

dopamine [4-(2-aminoethyl)benzene-1,2-diol, CAS (51-61-6)], epinephrine [(R)-4-(1-hydroxy-2-(methylamino)ethyl)benzene-1,2-diol, CAS (51-43-4)], norepinephrine [(R)-(-)-norepinephrine 1-1-(3,4-dihydroxyphenyl)-2-aminoethanol, CAS (51-41-2)], L-DOPA [L-3,4-dihydroxyphenylalanine, CAS (59-92-7)], DOPAC [2-(3,4-dihydroxyphenyl)acetic acid, CAS (102-32-9)], THP [tetrahydropapaveroline, 1-[(3,4-dihydroxyphenyl)methyl]-1,2,3,4-tetrahydro-6,7-isoquinolinediol; norlaudanosoline, CAS (47-99-3)], tyramine [4-(2-aminoethyl)phenol, CAS (51-67-2)].

2.8.3. Peptidic agonists and antagonists of opioid receptors

DAGO [D-Ala², NMe-Phe⁴, Gly^{-ol}]-enkephalin, CAS (78123-71-4)], CTOP [D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂, CAS (103429-31-8)], CTAP [D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂, CAS (103429-32-9)].

2.8.4. Semi-synthetic antagonists (structures close to morphine)

naloxonazine [Bis-(5 α -4,5-epoxy-3,14-dihydroxy-17-(2-propenyl)-morphinan-6-ylidene) hydrazine dihydrochloride, CAS (82824-01-9)], naltrexone [(5 α)-17-(cyclopropylmethyl)-4,5-epoxy-3,14-dihydromorphinan-6-one hydrochloride, CAS (16676-29-2)], naloxone [(5 α)-4,5-Epoxy-3,14-dihydro-17-(2-propenyl)-morphinan-6-one hydrochloride, CAS (357-08-4)], naloxone methiodide [(5 α ,17R)-4,5-Epoxy-3,14-dihydroxy-17-methyl-6-oxo-17-(2-propenyl)-morphinan-6-ium iodide, N-methylnaloxonium iodide, CAS (93302-47-7)], naltrindole [17-cyclopropylmethyl-6,7-dehydro-4,5-epoxy-3,14-dihydroxy-6,7,2',3'-indo-

TABLE 1 Cross reactivity and K values of the anti-M6G antibody

	K (nM)	CR	Approx. nM equivalent to 1 nM M6G
M6G	3.5	100.00%	1
O6G	0.75	—	—
M3,6G	7.3	47.40%	2.1
Codeine	252.3	1.37%	72.3
Hydrocodone	323.6	0.38%	266.2
Morphine	1,627.3	0.21%	468.3
M3G	2,424.5	0.14%	638.7
Thebaine	4,538.3	<0.1%	>1,000
Hydromorphone	7,575.3	<0.1%	>1,000
Norcodeine	17,030.5	<0.1%	>1,000
Normorphine	21,712.3	<0.1%	>1,000

lomorphic, CAS (111469-81-9)], 3-methoxymaltrexone [morphinan-6-one,17-(cyclopropylmethyl)-4,5-epoxy-14-hydroxy-3-methoxy-, (5 α)-, CAS (16617-07-5)], Naloxone-3-glucuronide [(5 α)-4,5-Epoxy-3,14-dihydro-17-(2-propenyl)morphinan-6-one 3-glucuronide, CAS (22135-79-1)].

2.8.5. Synthetic agonist of MOR

fentanyl [N-(1-(2-phenylethyl)-4-piperidinyl)-N-phenyl-propionamide, CAS (437-38-7)].

2.8.6. Other

estradiol-17-glucuronide [1,3,5(10)-Estratriene-3,17 β -diol 17-glucuronide, CAS (15087-02-2)].

The dissociation constants [K] of the antibody for an antigen were determined by sigmoidal fitting (KyPlot 2.0, Tokyo, Japan). Antibody cross-reactivities for the different compounds [CR = K(M6G)/K(Compound) $\times$ 100] and nM equivalent to 1 nM of M6G are presented in Table 1.

2.9. Patients

This study was approved by our institutional review board for human experimentation (CPP n08/62-protocol nVD1 EUDRACT n2008-A01345-50). After informed consent, samples were collected from patients requiring clinical laboratory analyses. Blood from 37 endogenous morphine-positive critically ill patients, 8 negative controls (healthy donors) and 5 morphine positive controls (patients with morphine-patch; without codeine prescription) were collected. The blood was collected in lithium-heparinate tubes (LH-PSTII 367378, 17 U/mL, BD) and

plasma was recovered after centrifugation (4°C, 1,500 g, 10 Min [18]). Collected plasma were immediately stored at -80°C and were only thawed once, 15 Min before ELISA or extraction. Plasma's morphine concentrations were quantified using a morphine-specific ELISA kit (Immalysis) as previously described [18], whereas our M6G-specific ELISA prototype was used to quantify M6G in plasma.

2.10. Plasma Preparation for LC-MS/MS Analysis

100 μ L of plasma was acidified up to 0.1% formic acid (v/v) and centrifuged (14,000 g, 30 Min). Supernatants were collected before solid phase extraction (SPE). SPE procedure was performed with a vacuum manifold. OASIS HLB SPE-cartridge (1 cc, 30 mg, Waters) was first activated with 1 mL of methanol and then washed with 2 mL of H₂O/formic acid 0.1% (v/v). Sample was loaded and the SPE-cartridge was washed with 2 mL of H₂O/formic acid 0.1% (v/v). Elution was performed manually with single use filter pipet tips (to avoid potential cross contamination due to sprays) with 500 μ L of acetonitrile/methanol (50/50, v/v). Eluates were collected in low binding 1.5 mL tubes and dried under vacuum before MS analysis (see below).

Different extractions cartridge have been tested for the recovery of M6G (OASIS HLB and MC-X; SOLA; Bond Elute Certify; Strata-X 33u Polymeric Reversed Phase, Strata-XL 100u Polymeric Phase, Strata C18-E, Strata SAX, Strata Screen-A, Strata X-AW 33u Polymeric Weak Anion, Strata-X-CW 33u Polymeric Weak Cation, Strata WCX, Strata-X-C 33u Polymeric Strong Cation, Strata-SL-C 100u Polymeric Strong Cation, Strata Screen-C, Strata-X-C 33u Polymeric Strong Cation; Chromabond Drug). For very low amounts of alkaloids (1 pmol), the best M6G recovery was observed for the OASIS HLB cartridge (82 $\pm$ 11%) whereas morphine recovery was 54 $\pm$ 8%.

2.11. LC-MS/MS Instrumentation and Analytical Conditions

LC-analyses were used to confirm the presence of morphine and M6G in the multiple reaction monitoring mode (MRM). Analyses were performed on a Dionex Ultimate 3000 HPLC system (Thermo Scientific, San Jose, CA) coupled with a triple quadrupole TSQ Vantage (Thermo Scientific). The system was controlled by Xcalibur v. 2.0 software (Thermo Scientific).

2.11.1. LC separation.

Plasma extracts were resuspended in 20 μ L of water, mixed for 1 Min, followed by the addition of 80 μ L of acetonitrile and shaken for 1 Min. Of this preparation, 20% was loaded into a trap column Amide-80 (10 mm length $\times$ 2 mm I.D., 5 μ m; TOSOH) at a flow rate of 5 μ L/min with a solution containing 80% (v/v) of acetonitrile and 20% H₂O. After 5 Min, the trap column was set on-line with an analytical column TSKgel Amide-80 (25 cm length $\times$ 1 mm I.D., 5 μ m; TOSOH) at 50°C. Morphine and M6G elution were performed by applying linear gradient of buffers A/B. Buffer A corresponded to 5 mM ammonium acetate with 0.15% of formic acid (v/v), whereas buffer B was 100 % acetonitrile. After 5 Min of 80% of buffer

B, a linear gradient of 80-10 % of solvent B at 50 μ L/min over 15 Min was applied and followed by a washing step (2 Min at 90% of solvent A) and an equilibration step (8 Min of 80% of buffer B).

Qualitative analysis and quantification were performed in Selected Monitoring Reaction Mode (SRM) using a TSQ Vantage triple quadrupole mass spectrometer. For ionization, 3,600 V of liquid junction voltage and 250°C capillary temperature were applied. The selectivity for both Q1 and Q3 was set to 0.7 Da (FWHM). The collision gas pressure of Q2 was set at 1.5 mTorr of argon. For Morphine and M6G, the selection of the monitored transitions and the optimization of the collision energy were preliminary and manually determined.

The transitions and the corresponding collision energies (CE) used for SRM were the following: m/z 286.1 $\rightarrow$ m/z 201.1 (CE = 24 eV), m/z 286.1 $\rightarrow$ m/z 181.1 (CE = 37 eV), m/z 286.1 $\rightarrow$ m/z 165.1 (CE = 38 eV), m/z 286.1 $\rightarrow$ m/z 157.1 (CE = 36 eV), and m/z 286.1 $\rightarrow$ m/z 152. (CE = 62 eV), for morphine, and m/z 462.1 $\rightarrow$ m/z 286.1 (CE = 29 eV), m/z 462.1 $\rightarrow$ m/z 229.1 (CE = 35 eV), m/z 462.1 $\rightarrow$ m/z 201.1 (CE = 43 eV), m/z 462.1 $\rightarrow$ m/z 165.1 (CE = 65 eV) for M6G. Identification of the compounds was based on precursor ion, selective fragment ions and retention times obtained for D3-morphine and D3-M6G internal standards.

3. Results

3.1. Synthesis of M6G-3-NH₂

A convergent synthesis was used to introduce a pendant arm on M6G phenol moiety. Condensation of bromoacetyl bromide on Fmoc mono-protected butanediamine led to the expected linker which was able to react on M6G in presence of potassium carbonate using ethanol 100% as a solvent. These conditions allowed to obtain M6G-3-NH₂ in one step as protective group Fmoc was also removed during the reaction. The M6G-3-NH₂ (supplementary files) was coupled to KLH by glutaraldehyde and injected to rabbit in presence of Freund's adjuvant.

3.2. ELISA and Antibody Selectivity

Using our M6G-3-KLH, we were successful to generate a highly selective polyclonal antibody with high affinity for M6G. We used this antibody to develop a competitive ELISA with the following analytical performances. The intra assay CV determined on 3 plasma samples with low (1.26 ng/mL), medium (3.13 ng/mL) and high (6.34 ng/mL) M6G levels ranged from 6 and 10%. The inter assay CV assessed on the same 3 samples measured in 9 different runs ranged from 10 to 13%. The analytical limit of detection (defined as a two-standard deviation of standard 0) was evaluated at 0.07 ng/mL (0.15 nM). The lower limit of quantification (LLQ), defined as the lowest concentration in human plasma that can be measured with an imprecision of 20%, was determined at 0.3 ng/mL (0.64 nM). A pool of plasma samples was also spiked with calibrators (0.64; 1.6; 4 and 10 g/mL) and the recovery was found to range

FIG 1

Competitive immunoassay ELISA assay characterizing the specificity of anti-M6G antibody for natural or synthetic morphine-like alkaloids, catecholamines and monoamine compounds. A decrease in optical density indicates that the antibody is blocked by the tested antigens. As shown on the graph, the anti-M6G antibody recognize morphine M6G and M-3,6-G with an high affinity. A weak cross reactivity is observed for codeine, morphine and M3G. Only substance having a cross reactivity are represented.

between 92 and 110%. Two different plasma samples were diluted in the ELISA dilution buffer and the recovery of the dilution tests were between 96% and 106%. These analytical performances compare well with those of commercial assays for morphine [16] and allow to quantify M6G in human plasma with a good accuracy and precision.

The specificity of the anti-M6G antibody was assessed using the competitive-ELISA protocol, as described in the Materials and Methods section. The cross-reactivity of the anti-M6G antibody was tested against 30 compounds, including M6G, morphine, M3G, endogenous morphine precursors (codeine, THP, dopamine, L-DOPA, DOPAC, etc.), as well as monoamines (Table 1; Fig. 1). Our anti-M6G antibody displayed a very high affinity for M6G ($K = 3.5$ nM) and codeine-6-glucuronide (C6G; $K = 0.75$ nM) and, to a lesser extent for morphine-3,6-glucuronide (M3,6G; $K = 7.3$ nM, CR=47%). A weaker affinity was determined for codeine ($K = 252.9$ nM, CR=1.37%), hydrocodone ($K = 923.6$ nM, CR=0.38%), morphine ($K = 1.62$ μ M, CR=0.21%) and M3G ($K = 2.42$ μ M, CR=0.14%). No or negligible cross-reactivity was found for other compounds (Table 1; Fig. 1). Together, these data demonstrate that the anti-M6G antibody properly detects M6G, M3,6G, and C6G without interfering with morphine, M3G and other monoamines and catecholamines. Exogenous codeine, in addition to being converted into morphine, is cleared *via* a glucuronidation process occurring mainly in the liver and leading to C6G [22,23]. So far, C6G has never been detected as an endogenous compound at rest and this is true also after morphine administration. M3,6G is a negligible compound as it

was only found at very low concentrations after exogenous morphine administration (for review see ref. 3).

3.3. Characterization of M6G in the Plasma by ELISA and Mass Spectrometry

Plasma samples from 39 patients were analyzed using our M6G-specific ELISA as well as LC-MS/MS. Plasma were collected from (i) healthy donors ($n = 8$), (ii) morphine-treated patients (morphine skin patch 12–48 H before blood collection, 20 mg/day, $n = 5$) and (iii) critically ill patients ($n = 26$), naive for morphine, but positive for eM when tested with a commercial morphine-specific ELISA [18].

In healthy donors (8 negative controls), eM and M6G were undetectable in plasma samples.

In morphine-treated patients (5 positive controls, naive for codeine), the mean of morphine concentration in plasma samples ranged from 108.7 to 640.7 nM (median=288.4 nM; 95% CI: 13.3, 563.1 nM), whereas values for M6G ranged from 324.6 to 2,062.6 nM (median=950.2 nM; 95% CI: -1,458.2, 3,358 nM).

In 26 selected morphine-positive critically ill patients, eM concentrations ranged between 0.7 and 149.4 nM in plasma samples (median=34.7 nM; 95% CI: 18.2, 50.8 nM), whereas M6G concentration ranged between 0.3 and 775.8 nM (median=150.8 nM; 95% CI: 63.2, 238.7 nM) in the same plasma samples. As previously described by us [18], eM levels were highly dependent of samples preparation, lithium-heparin plasma leading to a better eM recovery. In contrast, M6G quantification was only weakly affected by sampling procedure, as LH-plasma and serum only differ of 8 + 4% ($n = 18$; data not shown).

The absence or the presence of M6G was confirmed by a qualitative LC-MS/MS analysis. The M6G recovery using an acidic extraction followed by a OASIS HLB-SPE followed by a LC-MS/MS analysis led to with 32 + 11% of (see Materials and Methods section) when low amounts of M6G were used (1 pmole). LC-MS/MS analysis indicates that all samples from 8 negative controls were M6G-negative (8/8), whereas plasma all 5 positive controls were M6G-positive (5/5). Analysis of plasma of patients not treated with morphine showed that 73.0% of M6G-ELISA positive samples (19/26) contain M6G when analyzed by LC-MS/MS. Among the 27% left, 19.2% of ELISA's M6G-positive samples were below the LC-MS/MS detection limit (<100 fmole), whereas 7.7% of the samples were close to this detection limit.

4. Discussion

This study describes the production and the use of a new hapten to produce a specific anti-M6G antibody. However, strong cross-reactivities for C6G and, to a lesser extent, for M3,6G have been shown. Indeed, 50–70% of exogenous codeine is converted into C6G by UGT2B7, a process mainly occurring in the liver [22,23]. So far, no C6G has been found

after morphine administration ruling out a possible methylation of morphine into codeine and, therefore, the possible production of C6G. In addition, C6G has never been identified as a possible endogenous compound. M3,6G is a negligible compound as it was only found at very low concentrations after morphine injection (for review see ref. 3). Together, it appears that both C6G and M3,6G remained at negligible levels after morphine administration. However, our M6G-ELISA kit is to be avoided if codeine has been prescribed to patients.

This antibody was used to set up an ELISA kit that has been tested on a cohort of patients. Our results describe for the first time the presence of endogenous M6G in the plasma of critically ill patients. Hence, patients with elevated eM or exogenous morphine blood levels also display higher M6G levels. The presence of M6G was qualitatively confirmed by a LC-MS/MS analysis except for patients with low M6G levels (under the LC-MS/MS detection limit). The high specificity of this M6G antibody revealed that the use of this ELISA kit is relevant to screen M6G's presence in patient's blood for future diagnostic procedures.

At the present time, the mechanisms explaining eM and endogenous M6G increases in pathological conditions remain unclear. However, in critically ill patients (i.e., sepsis), eM elevation might represent a compensatory attempt to decrease inflammatory responses, as pathological levels are found in the nM range and inhibit IL-8 secretion from human polymorphonuclear cells [16].

In contrast to morphine, M6G binds MOR without interfering with TLR4 [24]. It is well stated that M6G has a higher analgesic potential than morphine [25,26]. Nevertheless, the level of the analgesia depends of both administration procedures and species (for review see refs. 3,27,28). It is acknowledge that M6G has a slightly lower affinity and a higher efficacy for MOR compared with morphine. However, morphine and M6G show more drastic differences as summarized by Wittner et al. [28]. Hence, compared with morphine, M6G has a lower volume of distribution and clearance rate, a lower permeability across the blood brain barrier and a slower clearance from the brain. The most attractive hypothesis for M6G differential effects is based on the presence of specific MOR splicing variants having higher affinity or specificity than morphine [29,30]. Interestingly, animal studies revealed that heroin and M6G act through receptors and mechanisms distinct from those of morphine. Hence, 3-methylnaltrexone selectively blocks the analgesic effects of heroin and M6G at doses that are inactive against morphine effects [30].

After an intravenous injection, M6G induced long-lasting analgesic effects in postoperative patients with limited respiratory depression and vomiting. These properties are likely to be related to its poor capacity to cross the BBB. M6G appears to be an effective substitutive opiate for the treatment of moderate to severe postoperative pain [31]. Therefore, phase III clinical trials in patients with postoperative pain has been performed [32] and have shown that increased doses of M6G are better tolerated than morphine (PAION, Aachen, Germany).

Together, our data further reveal the emerging potential of endogenous M6G as a pathological marker and we now provide a powerful tool to easily detect endogenous M6G levels. Based on M6G's effects, M6G levels in pathological conditions are likely to influence, positively or negatively, patient outcomes.

Acknowledgements

The authors thank Mrs. Elise Savier for her advices on the manuscript and Dr. Richard Hains for his help. This work was funded by Inserm, ANR Biotech, CNRS (Postdoctoral fellowship to A.L.-B.), University of Strasbourg, French Ministère délégué à la Recherche et à l'Enseignement Supérieur (Ph.D. fellowship to A.L.-B.), National Council for Scientific Research of Lebanon (CNRS-Lebanon to J.M.), and Fondation pour la Recherche Médicale (to Y.G.).

References

- [1] Mackenzie, P. I., Bock, K. W., Buchell, B., Guilleme, C., Koshiro, S., et al. (2005) Nomenclature update for the mammalian UDP glycosyltransferase (UGT) gene superfamily. *Pharmacogenet. Genom.* 15, 677-685.
- [2] Nagano, E., Yamada, H., and Oguni, K. (2000) Characteristic glucuronidation pattern of physiologic concentration of morphine in rat brain. *Life Sci.* 67, 2453-2464.
- [3] Laux Bichmann, Mouchéche, J., Verheze, J., and Gournon, Y. (2013) Endogenous morphine and its metabolites in mammals: history, synthesis, localization and perspectives. *Neuroscience* 238, 95-117.
- [4] Lewis, S. S., Hutchinson, M. R., Rezvani, N., Loran, L. C., Zhang, Y., et al. (2010) Evidence that intrathecal morphine 3-glucuronide may cause pain enhancement via toll like receptor 4/MD 2 and interleukin 1beta. *Neuroscience* 165, 589-593.
- [5] Goldstein, J. A., James, I. F., Lowmy, L. I., Weitz, C. J., et al. (1985) Morphine and other opiates from bovine brain and adrenal. *Proc. Natl. Acad. Sci. U S A* 82, 5203-5207.
- [6] Oka, K., Kawanishi, J. O., and Spector, S. (1985) Isolation of morphine from rod skin. *Proc. Natl. Acad. Sci. U S A* 82, 1852-1854.
- [7] Conner, J., Oka, K., Rossi, A., Rice, K. C., and Spector, S. (1985) Presence and formation of codeine and morphine in the rat. *Proc. Natl. Acad. Sci. U S A* 83, 4565-4567.
- [8] Weitz, C. J., Lowmy, L. I., Faull, K. F., Ristner, G., and Goldstein, A. (1985) Morphine and codeine from mammalian brain. *Proc. Natl. Acad. Sci. U S A* 83, 9794-9798.
- [9] Hazum, E., Sabatka, J. J., Chang, K. J., Brem, O. A., Findlay, J. W., et al. (1981) Morphine in cow and human milk could dietary morphine constitute a ligand for specific morphine (mu) receptors? *Science* 213, 1010-1012.
- [10] Boettcher, C., Fellemeier, M., Boettcher, C., Orger, B., and Zerk, M. H. (2005) How human neuroblastoma cells make morphine. *Proc. Natl. Acad. Sci. U S A* 102, 8435-8439.
- [11] Gournon, Y., Muller, A., Gaudard, E., Marban, C., Gasnier, C., et al. (2006) Identification of morphine 6-glucuronide in chromaffin cell secretory granules. *J. Biol. Chem.* 281, 8082-8088.
- [12] Muller, Gaudard, E., Talib, O., Kennel, V., Laux, A., et al. (2008) Endogenous morphine in SH-SY5Y cells and the mouse cerebellum. *PLoS One* 3, e1641.
- [13] Brix-Ovinsen, V., Tønnesen, E., Sanchez, R. G., Billings, T. V., and Sletano, G. B. (1997) Endogenous morphine levels increase following cardiac surgery as part of the anti-inflammatory response? *Int. J. Cardiol.* 62, 191-197.
- [14] Majesnik, W. J., Molina, P. E., and Abumrad, N. N. (1989) Mammalian opiate alkaloid synthesis: lessons derived from plant biochemistry. *Shock* 12, 165-173.

- [15] Yoshida, S., Ohno, J., Yamazaki, K., Kamei, H., Harada, Y., et al. (2000) Effect of surgical stress on endogenous morphine and enkephalin levels in the plasma after laparoscopic or open cholecystectomy. *Surg. Endosc.* 14, 137-140.
- [16] Glazard, E., Wehlers, I. O., Lavauze, T., Muller, A. H., Laux, A., et al. (2010) Endogenous morphine levels are increased in sepsis: a partial implication of neutrophils. *PLoS One* 5, e8791.
- [17] Madbouly, K. M., Senagore, A. J., and Olanrewaju, C. P. (2010) Endogenous morphine levels after laparoscopic versus open colectomy. *Br. J. Surg.* 97, 759-764.
- [18] Laux, Behlmann, Glatz, N., Mouheiche, J., Stuber, O., Wehlers, I. O., et al. (2012) Comparison of serum and lithium heparinate plasma for the accurate measurements of endogenous and exogenous morphine concentrations. *Br. J. Clin. Pharmacol.* 74, 381-388.
- [19] Manzoni, M. A., Uebay, E. O., Kozle, J., Munjal, I. O., and Spector, S. (1997) Endogenous codeine and morphine in anorexia and bulimia nervosa. *Life Sci.* 60, 1741-1747.
- [20] Chamon, G., Doudnikoff, E., Laux, A., Benhet, A., Porras, G., et al. (2011) Endogenous morphine-like compound immunoreactivity increases in parkinsonism. *Brain* 134, 2321-2338.
- [21] Mashayekhi, S. O., Ghandforoush Sabari, M., Routledge, P. A., and Hain, R. O. (2009) Pharmacokinetic and pharmacodynamic study of morphine and morphine 6 glucuronide after oral and intravenous administration of morphine in children with cancer. *Biopharm. Drug Dispos.* 30, 99-106.
- [22] Vree, T. B. and Verwey van Wissen, C. P. (1992) Pharmacokinetics and metabolism of codeine in humans. *Biopharm. Drug Dispos.* 13, 445-460.
- [23] Raungrut, P., Uthaisichat, V., Elliot, O.J., Janchawee, B., Somogyi, A. A., et al. (2010) In vivo in vivo extrapolation predicts drug-drug interactions arising from inhibition of codeine glucuronidation by dextropropoxyphene, fluconazole, ketoconazole, and meclizone in humans. *J. Pharmacol. Exp. Ther.* 334, 609-618.
- [24] Hutchinson, M. R., Zhang, Y., Shrivastava, M., Evans, J. H., Buchanan, M. M., et al. (2010) Evidence that opioids may have toll like receptor 4 and MD2 effects. *Brain Behav. Immun.* 24, 88-96.
- [25] Rawes, B., Gout, R., Carrelsson, G., Paveoni, E., and Cros, J. (1990) Morphine 6 glucuronide is more mu selective and potent in analgesic tests than morphine. *Prog. Clin. Biol. Res.* 328, 477-480.
- [26] Osborne, P. B., Chiang, B., and Christie, M. J. (2000) Morphine 6 beta glucuronide has a higher efficacy than morphine as a mu opioid receptor agonist in the rat locus coeruleus. *Br. J. Pharmacol.* 131, 1422-1428.
- [27] Lorsch, J. and Gossling, G. (2001) Morphine 6 glucuronide: an analgesic of the future? *Clin. Pharmacokinet.* 40, 495-498.
- [28] Whitaker, S. and Kern, S. E. (2006) Role of morphine's metabolites in analgesia: concepts and controversies. *Aggs. J.* 8, E348-E352.
- [29] Xu, J., Xu, M., Rossi, G. C., Pasternak, G. W., and Pan, Y. X. (2011) Identification and characterization of seven new exon 11 associated splice variants of the rat mu opioid receptor gene, OPRM1. *Mol. Pain* 7, 3.
- [30] Pasternak, G. W. (2012) Preclinical pharmacology and opioid combinations. *Pain Med.* 13, 54-511.
- [31] Smith, T. W., Binning, A. R., and Oshay, A. (2009) Efficacy and safety of morphine 6 glucuronide (M6G) for postoperative pain relief: a randomized, double blind study. *Sur. J. Pain.* 13, 238-239.
- [32] Joshi, G. P. (2008) Morphine 6 glucuronide, an active morphine metabolite for the potential treatment of postoperative pain. *Curr. Opin. Investig. Drugs* 9, 786-799.

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