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Development and validation of a radioreceptor assay for the determination of morphine and its active metabolites in serum

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Abstract

This article describes the development and validation of a radioreceptor assay for the determination of morphine and morphine-6-β-glucuronide (M6G) in serum. The assay is based on competitive inhibition of the μ-opioid-selective radiolabeled ligand [^{3}H]-DAMGO by opioid ligands (e.g. M6G) for binding to the striatal opioid receptor. The assay has been validated according to the Washington Conference Report on Analytical Method Validation. The radioreceptor assay can be performed in serum without prior pre-treatment of the sample. Direct addition of the sample results in no significant loss in maximal binding sites, and therefore, no loss in sensitivity. The assay proves to be selective for a multitude of opioid agonists and antagonists (e.g. morphine IC_{50} = 4.1 nM and M6G IC_{50} = 12.8 nM). Moreover, morphine-3-glucuronide (M3G) displays a low affinity (IC_{50} = 1100 nM) for the μ-opioid receptor and according to the literature demonstrates no analgesic activity. This makes discrimination, in relation to the analgesic effect, of the two metabolites of morphine possible. The assay is fast (assay time <4 h, analysis 5 min/sample), easy and the sensitivity (limit of detection (LOD) = 1.6 nM M6G-equivalents) is such that very potent agonists, like morphine and M6G, can be measured at the desired serum levels. The assay is accurate (<18%), but precision is limited if measured over several days (>35%). The assay is most accurate and precise if measured over a range from 3.5 to 40 nM M6G-equivalents. Based on the limited inter-assay precision, we propose to use this receptor assay mainly as a screening tool for neonates treated with morphine.

Keywords: Quantitative radioreceptor assay; μ-Opioid receptor; Morphine; Morphine-6-β-glucuronide; Morphine-3-glucuronide; Serum; Therapeutic drug monitoring; Validation

1. Introduction

The use of morphine as an analgesic in pre-term newborns (neonates, <37 weeks) is very common, due to the many painful procedures and stressful circumstances they undergo. Despite the fact that morphine is a powerful analgesic, its use is rather questionable [1,2]. Severe side effects, such as respiratory depression and seizures, are often occurring in these infants. Many correlation studies between respiratory side effects and plasma morphine concentrations have been performed to optimize the benefit to risk ratio of morphine [1,3]. The presence of metabolites of morphine like morphine-3-glucuronide (M3G) and morphine-6-β-glucuronide (M6G), seem to play an important role in the clinical effect of morphine, especially, in neonates [1,4,5]. Morphine glucuronidation on the third and sixth position gives rise to more polar compounds, which normally do not pass the blood–brain barrier and are easily excreted by the kidneys. Neonates differ from other children and adults in that they accumulate more of these morphine metabolites (poor renal excretion) despite a lower rate of production (immature liver). Next to an inadequate liver and kidney function, neonates also suffer from a poorly developed blood–brain barrier, which facilitates pen-

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etration of these polar metabolites into the brain [1,4,6]. An increased sensitivity of neonates to morphine may also be due to reduced protein binding. In neonates, only 20% of morphine is protein bound, which is significantly less than in adults (32%) [4,7].

The opioid receptor in the brain and mainly, the μ-opioid receptor, is responsible for the analgesia caused by morphine. The opioid receptor is primarily localized in the striatal tissue of the brain [8,9] and consists of three main subtypes (μ-, δ- and κ-opioids) in the ratio μ:δ:κ = 50:15:35 [10]. M6G binds to the μ-opioid receptor in the brain giving rise to more potent analgesia [11–14] and respiratory depression than morphine [1]. Evidence concerning the pharmacological activity of the predominant metabolite M3G is rather circumstantial [15,16]. According to the literature, M3G does not bind to the μ-opioid receptor [17–19], however, there is evidence that M3G antagonizes the analgesic effects of M6G and morphine, probably via a different receptor [16]. This antagonism leads to the assumption that neonates may be not as sensitive to respiratory depression as deduced from morphine and M6G levels only [5,20]. To overcome this problem introduced by morphine and its metabolites and because the kinetics and metabolism vary between individuals, it is necessary to control the blood levels. Maturation of the liver, improved renal function and closure of the blood–brain barrier result in the necessity of monitoring these levels every day in neonates [1,4,5,21].

Pain assessment in neonates is a difficult task, which makes a correlation between the extent of analgesia, pain perception and side effects of utmost importance [20]. Conventional methods, like immunoassays and chromatographic analyses are all used in therapeutic drug monitoring of morphine and its active metabolites. While these methods determine the concentration with sufficient accuracy and precision, they are not able to correlate this concentration with the pain response, since they do not measure the pharmacological effect at the receptor. The receptor assay is, thus, complementary to these methods, as it determines plasma levels in relation to the extent of receptor binding. The main advantage of using a receptor assay is that any metabolite, which is active, is determined in this assay. The receptor assay gives an overall concentration of μ-opioid binding activity, which takes higher affinity ligands and active metabolites proportionally more into consideration even if they are at lower concentrations. This concentration is likely to be better correlated to the analgesic effect in neonates than the concentration of individual ligands measured with chromatographic methods or the concentration of all morphine-related ligands measured using immunoassays.

In the following, we describe the development and validation of a direct radioreceptor assay for therapeutic drug monitoring of morphine-6-β-glucuronide in serum. While radioreceptor assays have been developed for other opiate narcotics like morphine and fentanyl [22–27], this is not the case for the important metabolite morphine-6-β-glucuronide. M6G has only been determined in biological samples using liquid chromatography [28–33] and as morphine equivalents with immunoassays [20,34].

2. Materials and methods

2.1. Reagents and materials

All chemicals were of analytical grade unless stated otherwise. Morphine-6-β-glucuronide was purchased from Lipomed (Arlesheim, Switzerland) and Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH (tyrosil-3,5-[^{3}H]-DAMGO; specific activity, 50.0 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA, USA). Bacitracine, foetal bovine serum and sodium hydroxide pellets were obtained from Fluka (Buchs, Switzerland), BioWhittaker (Verviers, Belgium) and Janssen Chimica (Beerse, Belgium), respectively. Copper(II) sulfate was bought from Brocacef (Maarssen, The Netherlands). Blank human serum was obtained from the Laboratory for Clinical and Forensic Toxicology and Drug Analysis, University Medical Centre Groningen (Groningen, The Netherlands). Other chemicals were purchased from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA), multiscreen FB opaque plates, 1.0 μm glass fiber type B filtration plates, a multiscreen assay system and a punching device were obtained from Millipore (Bedford, MA, USA). Polyethylene tubes and polyethylene counting vials were supplied by Greiner (Alphen a/d, Rijn, The Netherlands). Ultima Gold scintillation liquid was obtained from Packard Biosciences (Groningen, The Netherlands). Demineralized water was further purified by an Elgastat Maxima instrument (ELGA, High Wycombe, UK) before use.

2.2. Preparation of receptor suspension

Striata were dissected from calf brain and stored at −80 °C. After thawing, 4.5 g of striatal tissue was homogenized in 75 ml of 0.32 M sucrose in Tris–HCl (50 mM, pH 7.4), using a Glass–PTFE Potter–Elvehjem tissue homogenizer (type RZR2021, Heidolph Instruments GmbH & Co., Schwabach, Germany) at 1200 rpm. The cell nuclei were removed by centrifugation at 4 °C for 10 min at 600 × *g* in a Heraeus centrifuge type 4123 (Heraeus-Christ GmbH, Osterode, Germany). The supernatant was collected and the pellet was resuspended, homogenized and centrifuged in the same manner as described above. After collecting the second supernatant, the fractions were combined and ultracentrifuged at 100,000 × *g* at 4 °C for 45 min using an L8–55 ultracentrifuge and an SW28 rotor from Beckman Instruments (Mijdrecht, The Netherlands). After the centrifugation step, the pellet containing the cell membranes with the opioid receptors, was resuspended and homogenized in 150 ml Tris–HCl (50 mM, pH 7.4) and incubated for 30 min at 30 °C in order to eliminate endogenous enkephalins and endorphins. Cell membranes were precipitated (P2-pellet) by centrifugation for 20 min at 50,000 × *g* (Beckmann L8–55) [35].

The pellet was distributed in aliquots of 400 mg (wet weight) in cryogenic vials (Greiner, Alphen a/d, Rijn, The Netherlands) and snap-frozen in liquid nitrogen. The vials were stored at −20 °C.

2.3. Determination of the affinity of [^{3}H]-DAMGO and the receptor density of the tissue preparation (saturation curve)

After thawing, the P2-pellet was suspended and homogenized in Tris–HCl (50 mM, pH 7.4) containing 1 mM Na_2–EDTA, 10 mM $MgSO_4$, 1 mM benzamidine, 0.01% bacitracine and 0.002% soybean trypsin inhibitor [36] to a protein concentration of ∼2 mg/ml. The filters of a multiscreen FB filtration plate were moistened by pipetting 200 μl Tris–HCl (50 mM, pH 7.4) into each well and vacuum was applied through the multiscreen manifold. Saturation binding experiments were performed using 20 μl of the μ-opioid-selective agonist [^{3}H]-DAMGO (specific activity, 50 Ci/mmol) over a final concentration range of 0.1–10 nM in a total assay volume of 200 μl. Non-specific binding was determined in the presence of 20 μl of 10 μM final morphine-6-β-glucuronide. The receptor suspension was homogenized and incubated for 60 min at 30 °C [36]. After addition of 160 μl of membrane–receptor suspension, the plate was incubated for 30 min at 30 °C. The incubation was ended by filtration under reduced pressure, followed by addition of twice 200 μl of ice-cold Tris–HCl (50 mM, pH 7.4). The filters were transferred into polyethylene counting vials and 3.5 ml of Ultima Gold scintillation cocktail was added. Radioactivity was counted after shaking the vials for 2 h with a Packard scintillation counter (Tricarb, Downers Grove, IL, USA) for 5 min/vial. The maximal number of binding sites (B_{max}) and the equilibrium dissociation constant K_d for the labeled ligand were calculated with the EBDA–LIGAND computer fitting program (Version 4.0, Biosoft, Cambridge, UK). This program is based upon the LIGAND program originally developed by Munson and Rodbard [37].

2.4. Bioanalytical validation

The radioreceptor assay was validated in Tris–HCl (50 mM, pH 7.4) and foetal bovine serum (1:1 diluted with Tris–HCl (50 mM, pH 7.4)) by performing inhibition curves using a final concentration of 1.1 nM of the labeled ligand [^{3}H]-DAMGO, together with an increasing concentration of morphine-6-β-glucuronide or another ligand of the opioid receptor. Quality control (QC) serum samples spiked with morphine-6-β-glucuronide at approximately 20, 50 and 80% inhibition were included during each run to assess accuracy, intra- and inter-assay precision. Serum samples, collected from neonates treated with morphine, were diluted 1:1 with Tris–HCl (50 mM, pH 7.4) prior to analysis (serum samples were obtained from routine therapeutic drug monitoring at the University Medical Centre, Groningen). The assay procedure was performed as described in Section 2.3. The QC-samples were back-calculated from the calibration curve using Eq. (1) [25,38]. The IC_{50}-value was converted via the Cheng–Prusoff equation (Eq. (2)) [39] to the corresponding affinity constant K_i.

$$X = IC_{50} \times \left(\frac{T - Y}{Y - NS}\right)^{1/s} \quad (1)$$

where T is the total binding, NS the non-specific binding, $Y = [LR^*]_T - [LR^*]_{NS}$ (nM), IC_{50} the concentration of the inhibitor displacing 50% of bound labeled ligand, s the slope, $[LR^*]$ the concentration of ligand bound to the receptor and X is the amount of analyte in nM.

$$IC_{50} = K_i \times \left(1 + \frac{[L^*]}{K_d}\right) \quad (2)$$

where K_i is the affinity constant, $[L^*]$ the concentration of free labeled ligand and K_d is the dissociation constant.

The protein amount was determined using the method as described by Lowry et al. [40]. The Lowry method was, however, carried out after precipitation with a final concentration of 6% trichloroacetic acid to eliminate the interference of Tris [41].

3. Results and discussion

3.1. Saturation of striatal membranes with the μ-opioid peptide agonist [^{3}H]-DAMGO

Saturation binding experiments with the μ-opioid agonist [^{3}H]-DAMGO on a calf striatal tissue preparation gave maximal binding sites of 95 ± 19 fmol/mg of protein. The binding was measured in Tris–HCl (50 mM, pH 7.4) and the calculated dissociation constant K_d was 0.80 ± 0.08 nM ($N = 2$, duplicate determinations). No significant decrease of binding properties was shown, when a saturation curve was performed in foetal bovine serum diluted 1:1 with Tris–HCl buffer (K_d of 0.77 nM, B_{max} of 114 fmol/mg protein). The saturation curves are depicted in Fig. 1A and the corresponding Scatchard plots are presented in Fig. 1B. The non-specific binding of the tritium labeled ligand was less than 16% of the total binding at a ligand concentration ranging from 0.5 to 2× K_d, as used in the competition experiments for measurements in buffer and serum, respectively.

In this assay, the opioid peptide agonist [^{3}H]-DAMGO is used as the labeled ligand due to its high affinity and selectivity [42] towards the μ-opioid receptor. The K_d-values found in buffer and 1:1 diluted serum correspond in average to the values found in literature for tritium labeled DAMGO. The B_{max}-values were, however, slightly lower if compared to the literature (B_{max} = 142 fmol/mg protein), where bovine striatal tissue was used as the receptor source [10,43].

3.2. Selectivity of the radioreceptor assay

A wide range of structurally different opioid agonists and antagonists were tested to characterize the binding profile of

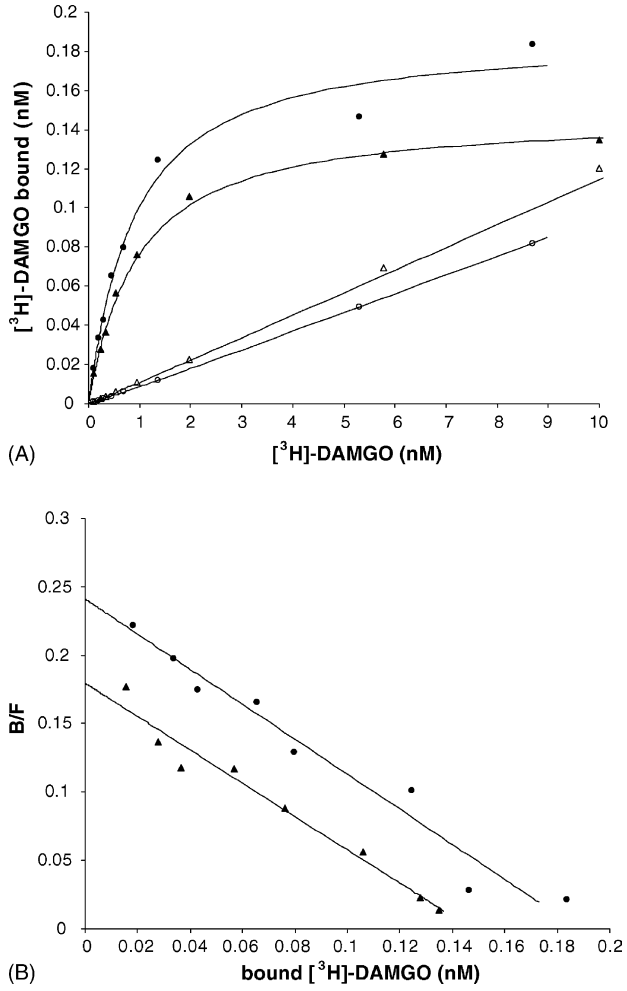

Fig. 1. (A) Saturation binding curves of specifically bound [^{3}H]-DAMGO determined in Tris–HCl (50 mM, pH 7.4) (▲; $N = 2$, duplicate) and foetal bovine serum diluted 1:1 with Tris–HCl (●; $N = 1$, duplicate) in a concentration range of 0.1–10 nM. Non-specific binding was determined in the presence of 100 μM morphine-6-β-glucuronide and is represented by the linear curves (Tris–HCl (△); serum:Tris–HCl = 1:1 (◯)). (B) Scatchard transformation.

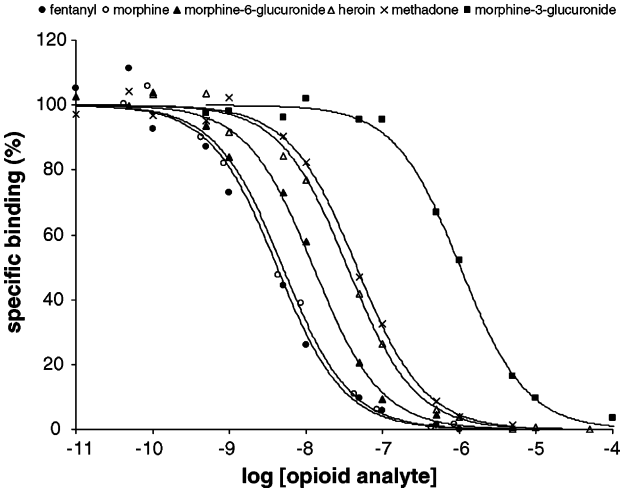

Fig. 2. Inhibition curves of different opioid agonists and antagonists measured in Tris–HCl buffer. Cyclazocine and nalorphine have affinity constants in the range of fentanyl, and therefore, the curves of these opioids are not displayed. The final concentration of the labeled ligand [^{3}H]-DAMGO was 1.1 nM. IC_{50}-values, K_i-values and the relative binding affinities are given in Table 1 (the relative binding affinities listed are in relation to morphine-6-β-glucuronide, which is set to 1).

the receptor. The assay proved to be selective for these μ-opioid receptor agonists and antagonists, which is depicted in Fig. 2. Table 1 shows the IC_{50}-values, together with the calculated K_i-values (via the Cheng–Prusoff equation [39]) and the relative binding affinities (RBA). The affinity constants are in the following rank order: fentanyl < cyclazocine < nalorphine < morphine < M6G < heroin < methadone < M3G.

The differences in affinities found in our work with some of the literature data (Table 1) may be due to the use of labeled ligands with different selectivity profiles than DAMGO [44]. Displacement of the μ-selective opioid agonist DAMGO by other opioid agonists or antagonists proceeds predominantly at the μ-opioid receptor site, giving rise to changes in measured affinities if less selective opioid labeled ligands are used, like naloxone. Nevertheless, if compared to the litera-

Table 1
Pharmacological binding profile of the μ-opioid receptor from calf striatal tissue measured in Tris–HCl buffer in relation to published data

Analyte	Property	IC_{50} (nM)	K_i (nM)[a]	RBA	K_i (nM) [reference]
Morphine-6-β-glucuronide	Agonist	12.8	5.39	1.00	10.0[b] [45], 0.6[c] [54]
Morphine	Agonist	4.09	1.89[d]	2.85	4.1[b] [45], 2.4[43]
Morphine-3-glucuronide	Antagonist	1100	463	0.01	766 [18], 37.1[c] [54]
Heroin (diamorphine)	Agonist	34.1	14.4	0.37	9.6[c] [54]
Cyclazocine	Agonist	3.17	1.33	4.05	0.23 [55], 0.9[b] [48]
Fentanyl	Agonist	2.7	1.25[d]	4.31	1.2 [46], 2.92 [56]
Methadone	Agonist	46.6	19.6	0.28	28.8[c] [54]
Nalorphine	Antagonist	3.5	1.47	3.67	1.2 [57], 2.7 [55]

IC_{50}-values were obtained from the corresponding inhibition curves and were converted to inhibition constants (K_i) via the Cheng–Prusoff equation [39].
[a] $K_d = 0.8$ nM and $[L^*] = 1.1$ nM.
[b] IC_{50}-value.
[c] Pre-incubated with [^{3}H]-DAMGO.
[d] $[L^*] = 0.93$ nM.

ture values, where DAMGO was used as the tracer ligand, the IC_{50} and K_i-values correspond very well [45,46]. Moreover, differences in the used tissues and the set-up of the radioreceptor assay (e.g. pre-incubation of the labeled ligand with the receptor preparation [47]) may have contributed to the differences found in the affinities as well.

Relative binding affinities were calculated as the ratio of the K_i-values of the different individual opioid ligands for the μ-opioid receptor, where the RBA value of morphine-6-β-glucuronide was arbitrarily set to 1 (Eq. (3)). The receptor assay measures the sum of all the μ-opioid active ligands present, which makes the determination of the relative binding affinities necessary to determine the contribution of the different opioid ligands to the overall value and to make comparison with chromatographic techniques possible. It should be noted that in the determination of opioid activity in serum of neonates, the signal is only reflected by the presence of morphine and M6G, as glucuronidation is the main metabolic pathway (60–75%, with 10% leading to M6G [30]). Despite the fact that M3G is the main metabolite, it possesses a very low affinity. Moreover, other morphine metabolites, such as codeine, morphine-3-ethereal sulfate and normorphine, possess either less affinity and/or are produced in negligible quantities and will, therefore, not largely contribute to the observed signal.

$$\text{RBA} = \frac{K_i\,(\text{M6G})}{K_i\,((\text{ant})\text{agonist})} \quad (3)$$

3.3. Application of the radioreceptor assay in serum

Our radioreceptor assay did not require any sample pretreatment for determinations in serum except for a 1:1 dilution in buffer. No interference from serum proteins (Table 2) on the maximal amount of labeled ligand specifically bound to the receptor B^*_{max} (*t*-test, $\alpha = 0.05$, $P = 0.79$ and 0.74 for foetal bovine and human serum, respectively) was observed during the development of this assay. Also, no shift of the analytes to a lower affinity as a consequence of serum proteins and/or the presence of sodium ions was shown. The affinity constants of, e.g. morphine and fentanyl measured in foetal bovine serum were the same as measured in buffer (results not shown), except for the affinity of morphine-6-β-glucuronide. As depicted in Table 2, the affinity of morphine-6-β-glucuronide shifts to a significantly (*t*-test, $\alpha = 0.05$, $P = 0.009$) lower value if measured in foetal bovine serum. The affinity of morphine-6-β-glucuronide in human serum does not change significantly (*t*-test, $\alpha = 0.05$, $P = 0.22$). It is, thus, advised to multiply the observed data, back-calculated from the calibration curve made up in foetal bovine serum, by a factor of 2. Another option is to back-calculate opiate activity from neonatal samples using a calibration curve made up in blank human serum. The reason why morphine-6-β-glucuronide shifts to a higher affinity if measured in foetal bovine serum remains to be elucidated. Based on ethical grounds, the assay was validated using foetal bovine serum.

Thus, a direct binding assay can be performed, if the labeled ligand is not excessively influenced by matrix components, like metal ions (serum contains 140 mM Na^+ ions), lipids and serum proteins. Especially, sodium, which is present during this assay in a final concentration of 7 mM, is known to reduce the binding affinity of μ-opioid agonists, while increasing the binding affinity of μ-opioid antagonists [25,48]. The opposite is true for divalent cations, like manganese, which can counteract the effects of sodium, as discussed by Levi et al. [25]. Also magnesium is known for its positive effect on the agonist affinity state of the receptor [49]. We, therefore, established a binding buffer containing 10 mM $MgSO_4$ to counteract the effect of sodium in serum samples (see Section 2) and propose to perform a direct assay, also because of the small serum volumes that are obtained from neonates. Advantages of this direct radioreceptor assay [35] are the simplicity, the minimal losses of drug compounds and no requirement of a labour intensive and costly sample preparation step, like solid-phase extraction (SPE). Moreover, metabolites, which may not be extracted using SPE or liquid–liquid extraction (LLE) will not be missed if this direct assay is performed.

3.4. Freeze–thaw stability of the receptor preparation

Stability of the μ-opioid receptor to freeze–thaw cycles was evaluated by performing one, two and three cycles followed by determining the decrease in specific binding. The receptor was frozen at −20°C for 2 h and thawed at room temperature. The maximal amount of labeled ligand specifically bound to the receptor B^*_{max} was not affected by two ($B^*_{max} = 77.0$ fmol/mg protein) or three ($B^*_{max} = 81.3$ fmol/mg protein) freeze–thaw cycles in comparison with one freeze–thaw cycle ($B^*_{max} = 68.0$ fmol/mg protein). There is also no loss in affinity towards the receptor

Table 2
Binding properties and assay characteristics in buffer and serum ($N = 5$, duplicate)

	IC_{50} (nM)	K_i (nM)	B^*_{max} (pmol/mg protein)[a]	LOD (nM)	Goodness of fit, R^2
Tris–HCl	15.5 ± 4.5	6.7 ± 2.4	0.063 ± 0.025	5.0	0.9959
Foetal bovine serum	9.0 ± 2.5	2.8 ± 0.9	0.058 ± 0.029	1.6	0.9984
Human serum	14.0 ± 4.9	5.0 ± 1.6	0.072 ± 0.004	2.4	0.9953

Morphine-6-β-glucuronide was determined in Tris–HCl buffer (50 mM, pH 7.4) and 1:1 diluted foetal bovine and human serum, using [^{3}H]-DAMGO as the labeled ligand and striatal tissue containing the μ-opioid receptor.

[a] B^*_{max}, maximal amount of labeled ligand specifically bound to the receptor, as determined in competition binding experiments.

Table 3
Accuracy and precision of measuring morphine-6-β-glucuronide in foetal bovine serum ($N = 5$)

[M6G] (nM)	Back-calculated [M6G] (nM)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
3	2.5	82.3	36	49
9	8.7	96.8	23	35
65	58.2	89.5	19	51

Morphine-6-β-glucuronide was spiked in 1:1 diluted foetal bovine serum at the lower end (3 nM) and upper end (65 nM) of the curve (3 nM) and at the IC_{50}-value (9 nM). The back-calculation to morphine-6-β-glucuronide equivalents was done using the least-squares regression equation that described the inhibition curve (Eq. (1), [25,38]).

with IC_{50}-values for morphine-6-β-glucuronide of 9.7, 7.9 and 8.7 nM for one, two and three freeze–thaw cycles, respectively. The non-specific binding averaged around 18% at a ligand concentration of 1.4 nM, which is acceptable for further assay development. It is convenient that the receptor suspension is stable to at least three cycles of freeze–thawing. It is, however, recommended to avoid such freeze–thaw cycles as much as possible.

3.5. Sensitivity, precision and accuracy

The sensitivity of a receptor assay is determined by the concentration and the affinity of both the displacing ligand and the labeled ligand. Next to this, the specific activity of the radioligand, the receptor concentration and the incubation volume determine the overall sensitivity of the assay [50]. The sensitivity can be assessed by calculating the limit of detection (LOD), where the LOD is defined as the lowest concentration of displacing ligand that can be significantly differentiated from the background noise [51]. After measuring the zero standard (amount of bound labeled ligand in the absence of analyte) repeatedly, the data were statistically treated to obtain a standard deviation (S.D.). The concentrations at which $-2\times$ S.D. intersected the calibration curve represented the LOD-values [50], which were 5.0 nM (2.3 ng/ml), 1.6 nM (0.7 ng/ml) and 2.4 nM (1.1 ng/ml) M6G-equivalents for Tris–HCl buffer, foetal bovine and human serum, respectively. The LOD-values are summarized in Table 2. In this table, the goodness of fit is given, which relates to the appropriate choice of the curve-fitting model. The fit procedure proved to give high correlation coefficients both in buffer and serum of $R^2 = 0.9959$ and >0.995, respectively. As stated by Findlay et al. [52], the correlation coefficient is, however, too restrictive in model assessment and should, therefore, be accompanied by the evaluation of the lack of fit (% relative bias, R.E. ± 10%). The mean relative bias defined as the relative bias between back-calculated and nominal concentrations of the calibration samples, was determined as no more than 10% over the calibration range of the described assay.

Accuracy and precision (Table 3) are based on the analysis of spiked foetal bovine serum containing morphine-6-β-glucuronide in known concentrations (quality control samples) and were calculated using one-way analysis of variance (ANOVA) at a 95% confidence interval. The concentrations are chosen near the lower and upper end and at the mid-range of the calibration curve, around the IC_{50}-value. The accuracy values at 3, 9 and 65 nM all fall within the 20% criteria for biological assays. Shah et al. [51] and Findley et al. [52] even state that the accuracy can be set to 25% at the lower and the higher end of the calibration curve, due to the greater imprecision of receptor assays in comparison with chromatographic methods. The precision of the assay should also not exceed 20%, with values less than 25% at the extremities of the calibration curve. The intra-assay precision (within-run) values were 36, 23 and 19% at the 3, 9 and 65 nM morphine-6-β-glucuronide concentrations. At the lower end the precision exceeds the acceptance range of 25%. The inter-assay (between-run) precision values at 3, 9 and 65 nM were 49, 35 and 51%, respectively, which limits the reproducibility of the assay when performed on a day-to-day basis. The QC-sample acceptance criteria of ±25% for accuracy, which means that 67% of all measured QC-samples fall within this range, has been met [51]. More than 73% ($N = 30$) fell within this range at the QC-levels of 9 and 65 nM. The assay is, therefore, most accurate and precise if measured for concentrations of unlabeled ligand displacing bound radioligand in the linear part of the curve (made up in foetal bovine serum), which approximately ranges from 3.5 to 40 nM (1.6–18.5 ng/ml).

The lack of day-to-day reproducibility is mainly due to the relatively low concentration of μ-opioid receptors present in striatal tissue. Addition of more receptor suspension negatively effects the labeled ligand affinity and thereby, the sensitivity of the assay due to underestimation of non-specific binding as a consequence of loss of proteins during filtration and subsequent washings. Moreover, the maximum capacity of the filters may be exceeded, which leads to a non-linear protein dependence of specific ligand–receptor binding [53]. To demonstrate the application of this radioreceptor assay to real samples, 10 serum samples obtained from neonates treated with morphine were measured (results not shown). Nine out of the 10 samples fell within the linear part of the calibration curve (3.5–40 nM) with the lowest value being 3.8 nM and the highest value being 38 nM. In one sample, no opioid activity could be measured. Based on the limited precision of the assay on a day-to-day basis of the calibration curve, we propose, however, to use this receptor assay mainly as a screening tool for neonates treated with morphine.

Table 4
Overview of radioreceptor assays that have been developed for the determination of narcotics in biological matrices

Analyte(s)	Labeled ligand	Matrix	Sample pre-treatment	Validation CV (%)	LOD (ng/ml)	NSB (%)	Reference
Fentanyl	[^{3}H]-Fentanyl (μ-agonist)	Urine	No	Intra: <12 Inter: <15	0.05	25–35	[26]
Buprenorphine Naloxone Morphine Fentanyl	[^{3}H]-Buprenorphine (partial μ-agonist, κ-antagonist)	Plasma and CSF	Yes, LLE	No	0.05	ND	[23]
Benzomorphan derivatives	[^{3}H]-Dihydromorphine (μ-agonist)	Plasma	Yes, LLE	No	ND	ND	[22]
Morphine Oxycodone M6G[a]	[^{3}H]-Dihydromorphine (μ-agonist)	Plasma	Yes, LLE	Intra: <9	ND	25	[44]
Fentanyl Pentazocine Morphine	[^{3}H]-Naloxone (μ-antagonist)	Serum	Yes, LLE Yes, LLE No	Inter: <15 – Inter: <29	0.3 3 5	20	[24]
Fentanyl Morphine	[^{3}H]-Naloxone (μ-antagonist)	Serum	No	Inter: <15	3 6	ND	[25]

All the radioreceptor assays made use of rat brain as the receptor source. CV, coefficient of variance; LOD, limit of detection; NSB, non-specific binding; CSF, cerebrospinal fluid; LLE, liquid–liquid extraction; ND, not determined.
[a] Could not be extracted using ether extraction pH 9 or 7.4.

4. Conclusions and perspectives

A range of assays has been developed for the determination of morphine or morphine analogs in biological matrices (see Table 4). Only Kalso et al. [44] reported the assay of morphine, morphine-6-β-glucuronide and morphine-3-glucuronide in plasma. Morphine-6-β-glucuronide could, however, not be determined in the radioreceptor assay, which is attributable to the loss of M6G during liquid–liquid extraction. A radioreceptor assay without a sample pre-treatment step would, therefore, be preferred.

In the assays of Levi et al. [25] and Alburges et al. [26] a radioreceptor assay without prior sample pre-treatment was executed. Levi et al. [25] studied fentanyl and morphine metabolism in human serum and Alburges et al. [26] described the validation of a direct radioreceptor assay of fentanyl in urine. The levels of morphine-6-β-glucuronide in urine are, however, not of pharmacological interest. Correlation with the pharmacodynamic effect is difficult, due to accumulation of this metabolite in urine. The procedure as described by Levi et al. [25] made use of the less μ-opioid-selective antagonist [^{3}H]-naloxone in a time consuming low-throughput format. Moreover, the use of [^{3}H]-naloxone can label both high- and low-affinity μ-opioid binding sites and is, therefore, less useful for reliable quantitation purposes if agonists are being measured [35]. Our work was aimed at the development and validation of a direct radioreceptor assay for morphine and its active metabolite morphine-6-β-glucuronide in serum utilizing the 96-well plate format, which makes automation possible.

DAMGO was used as the radiolabeled ligand, due to its more than 800-fold selectivity for the μ-opioid receptor over the δ- and κ-opioid receptor sites and its agonistic nature [42]. The displacement caused by opioid agonists and antagonists can, thus, be primarily attributed to the interaction with the μ-opioid receptor, which is the receptor subtype mainly involved in analgesia. The validated radioreceptor assay in foetal bovine serum proved to be saturable, selective, sensitive (LOD of 1.6 nM M6G-equivalents), fast (assay time <4 h, analysis 5 min/sample), easy and accurate (<18%). The selectivity is such that the other metabolite of morphine, morphine-3-glucuronide, does not display a high affinity towards the μ-opioid receptor and is, based on the relative binding affinities of the morphine analogs, no real source of interference in the radioreceptor assay. Moreover, as described by Bartlett and Smith [18], any binding of morphine-3-glucuronide towards the μ-opioid receptor found in vitro is likely due to morphine impurities.

The assay was validated according to the updated Washington Conference report by Shah et al. [51] and fulfilled all quality criteria except for a limited day-to-day reproducibility. This can likely be attributed to a low level of specific receptor binding sites (∼0.1 pmol/mg protein) in striatal tissue, which was the source of our receptor preparation. Increase in the number of maximal binding sites (B_{max})/mg protein and thereby, a likely increase in precision of the assay may be achieved by recombinant expression of the μ-opioid receptor in a suitable host-organism.

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