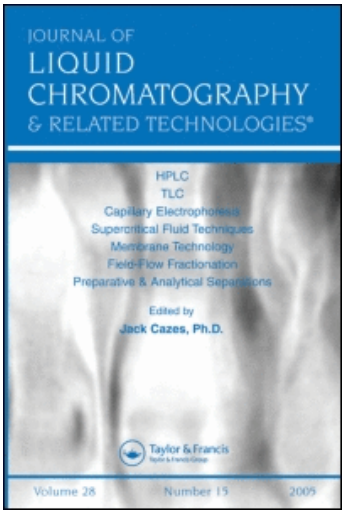

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A Stereospecific Microassay for the Determination of Morphine-6-β-D-Glucuronide and other Active Morphine Metabolites in the Neonatal Guinea Pig

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A STEREOSPECIFIC MICROASSAY FOR THE DETERMINATION OF MORPHINE-6-β-D-GLUCURONIDE AND OTHER ACTIVE MORPHINE METABOLITES IN THE NEONATAL GUINEA PIG

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ABSTRACT

A stereospecific microassay for the simultaneous determination of morphine and active metabolites morphine-6-β-D-glucuronide, morphine-3-β-D-glucuronide and normorphine using reversed-phase high-performance liquid-chromatography with ultraviolet detection is described. Solid-phase extraction was followed by chromatography on an octadecylsilica column eluted by 16.5% acetonitrile in phosphate buffer, pH 2.1 with sodium dodecyl sulfate as an ion-pairing agent. Using an 80 μl sample size the limits of quantitation were 94 ng/ml for all drugs. Coefficients of variation ranged from 2.0% to 6.3%. The assay is specific and separates the stereoisomers morphine-6-α-D-glucuronide and morphine-6-β-D-glucuronide. This microassay has been applied to samples of guinea pig plasma, following subcutaneous injections of morphine, and to hepatic microsomal enzyme preparations from a pregnant guinea pig. Neonatal guinea pigs produce substantial amounts of morphine-3-glucuronide and morphine-6-glucuronide from morphine *in vivo*, but normorphine was not detectable, whereas the liver microsomal enzyme preparations from a pregnant dam rapidly produced normorphine.

INTRODUCTION

Morphine is metabolized in man via at least two routes that produce active metabolites: conjugation with glucuronic acid at the three or six carbon, and N-demethylation at the 17 position[1-3]. Heroin (3,6-diacetylmorphine), which undergoes *in vivo* deacetylation to morphine[4,5], is also transformed to these active metabolites. Morphine-3-β-D-glucuronide's (M3G) activity is stimulatory in nature and M3G has been shown to causes seizures[13,14]. Normorphine (NOR), the N-demethylated metabolite of morphine, has activities similar to morphine[11,12], but at a reduced potency. Recently, considerable interest has arisen in the opioid activity of morphine-6-β-D-glucuronide (M6G) which has analgesic[6-8], respiratory depressant[9] and reinforcing properties[10] similar to morphine, but in these respects M6G is more potent than the parent compound. Very few data are currently available on the developmental pharmacology of these active compounds. However, morphine is used as an analgesic in neonatal and pediatric medicine[15] and *in utero* exposure to these metabolites may occur following the use of obstetrical analgesia or maternal heroin or morphine abuse.

Several assays for morphine and at least one metabolite, using high-performance liquid-chromatography (HPLC) with ultra-violet (UVD), electro-chemical (ECD) or fluorescence detection have been reported[16-23]. Assays using ECD, however, are unable to detect the M3G molecule[17-

19,23] as it lacks an oxidizable phenolic hydroxyl group. To counter this, either UVD or fluorescence detection has been used in line with ECD to detect M3G[17-19]. A limit to the usefulness of these assays still exists, though, due to the large sample sizes required (500-1000 μl of plasma) which are incompatible with the sample sizes available from small animals such as the guinea pig used in developmental pharmacology studies. The use of fluorescence detection or UVD allows the simultaneous determination of morphine and the active metabolites[16,20-22], but the assays available are again limited by the large sample volumes required in these methods (400-1000 μl). Many of these methods also use a time consuming extraction procedure processing only one sample at a time[16-18,22,23] and some require a double extraction process[16-18].

A microassay (an assay utilizing small sample volumes) for the determination of morphine and its active metabolites, M6G, M3G and NOR *in guinea pig plasma* has been developed. The procedure uses hydromorphone as the internal standard, an 80 μl sample size and a convenient, single-step detection with the readily available UVD technology. A rapid solid phase extraction allows simultaneous preparation of up to ten samples. The method is sensitive and specific for M6G, M3G, NOR and morphine, and is even able to separate the isomers morphine-6-β-D-glucuronide (referred to as M6G) and morphine-6-α-D-glucuronide. While the beta configuration is produced *in vivo* in mammals, the alpha isomer

has been reported as a contaminant of standards synthesized for HPLC[14]. The stereospecificity of the assay therefore adds a potential quality control application which has not been addressed by other methods[16-23]. Plasma samples from neonatal guinea pigs treated with morphine subcutaneously (s.c.) and the *in vitro* production of NOR from morphine in hepatic microsomal incubations have been examined using this microassay.

MATERIALS

Morphine•SO_4, morphine-6-α-D-glucuronide, morphine-6-β-D-glucuronide, M3G, NOR•HCl, nalorphine•HCl, dihydromorphine, morphine-3-ethereal sulfate, amphetamine, benzoylecgonine, benzoylnorecgonine and norcocaine were obtained from the Research Triangle Institute (Research Triangle Park, NC, U.S.A.) through the National Institute on Drug Abuse (Bethesda, MD, U.S.A.). Hydromorphone, naloxone•HCl, nalorphine•HCl, apomorphine and cocaine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Nalbuphine•HCl, oxymorphone•HCl, oxycodone•HCl and hydrocodone bitartrate were a gift from DuPont Pharmaceuticals (Wilmington, DE, U.S.A.). Codeine was a gift from Merck & Co. (Rahway, NJ, U.S.A.). l-Methadone•HCl was a gift from Eli Lilly and Company (Indianapolis, IN, U.S.A.). Stock solutions of 100 ng/μl were prepared by dissolving morphine, M6G, M3G, NOR and hydromorphone in water and

were stored in 1.5 ml aliquots at -15°C until use. Acetonitrile and methanol (Malinckrodt, Paris, KY, U.S.A.) were HPLC grade. Water was purified with a Milli-Q Plus Water System for analytical applications (Millipore Corp., Bedford, MA, U.S.A.). Sodium dihydrogen phosphate (monohydrate) (J.T. Baker Co., Phillipsburg, NJ, U.S.A.) and sodium dodecyl sulfate (SDS, Sigma Chemical Co.) were chromatography grade. All other chemicals were reagent grade.

METHODS

Liquid Chromatography

A Model 334 liquid chromatography system (Beckman Instruments, Berkeley, CA, U.S.A.) in line with a Model AS-100 HRLC automatic sampling system (Bio-Rad, Richmond, CA, U.S.A.), a 100 mm X 3.2 mm I.D., 3 μm particle size, octadecylsilica coated reversed-phase cartridge column (Velosep RP-18, Applied Biosystems, San Jose, CA, U.S.A.) and a Model 783A programmable absorbance detector (Applied Biosystems, Ramsey, NJ, U.S.A.) set at 214 nm and 0.002 absorbance units full scale was used to analyze extracted samples reconstituted in mobile phase. The detector response was integrated by a Model 3396 II integrator (Hewlett-Packard, Los Angeles, CA, U.S.A.) and stored on a computer using PEAK-96 software (Hewlett-Packard). Mobile phase was a modification of that reported by Svensson et al.[16], and consisted of 10 mM NaH_2PO_4, pH 2.1 with 1 mM SDS as an ion pairing agent and 16.5% (v/v) acetonitrile. The flow rate was 1.5 ml/min and the injection volume was 100 μl.

Solid Phase Extraction

Sample purification was accomplished using a C-18 Bond Elut extraction column with a 50 mg sorbent bed attached to a Vac Elut manifold (Varian Associates, Harbor City, CA, U.S.A.). After pretreating the column with 2 ml methanol and 2 ml water, an 80 μl sample was added to 720 μl of 0.5 M ammonium sulfate, pH 9.3, containing 675 ng of hydromorphone as the internal standard, in the reservoir of the Bond Elut. Vacuum was applied, and the column was washed with 3 ml of 10 mM ammonium sulfate, pH 9.3 and 1 ml of dichloromethane at an average flow of 1.5 ml/min. Elution was with 300 μl of 90:10 methanol:dichloromethane acidified with 2% (v/v) 1.0 N HCl. The eluate was dried at 45°C under nitrogen and samples were reconstituted in 300 μl of mobile phase. Standard curves were constructed from the analysis of plasma spiked with 94 to 3000 ng/ml of morphine, M6G, M3G and NOR. The ratio of morphine or metabolite peak area to the internal standard peak area was plotted against the concentration of drug in plasma to produce standard curves.

Animals

Dunkin-Hartley guinea pigs were purchased pregnant (Simonsen Labs, Gilroy, CA, U.S.A.), and parturition occurred approximately two weeks after arrival (neonatal studies) or were time-bred on site (microsomal studies). Animals were housed indoors with controlled light cycles, continuous ad libitum food and water, and daily health checks. The Animal

Care Department is fully accredited by the American Association for Accreditation of Laboratory Animal Care. The Department operates in compliance with the Animal Care Act and has an assurance letter on file at the National Institutes of Health. Experiments were carried out on 15-17 day old neonates. Three pups of two dams were dosed with 15 mg/kg morphine s.c. and blood samples were drawn into heparinized syringes via cardiac puncture following ether anesthesia 35-40 min after injection. Whole blood was centrifuged at 4°C and plasma was stored in polypropylene microfuge tubes at -15°C until analysis.

Microsomal Preparation and Incubation

Hepatic microsomes were prepared from a pregnant Dunkin-Hartley guinea pig on day 60 of gestation (term is 69 days) by the method of Williams et al.[24] and protein was determined by the method of Lowry et al.[25]. Morphine (1 mM) was incubated with 0.5 mg microsomal protein according to the method of Williams et al.[24].

Statistical Analysis

Least-squares linear regression analysis was used to determine standard curves for the assay, and to determine the initial rate of NOR production by hepatic microsomes.

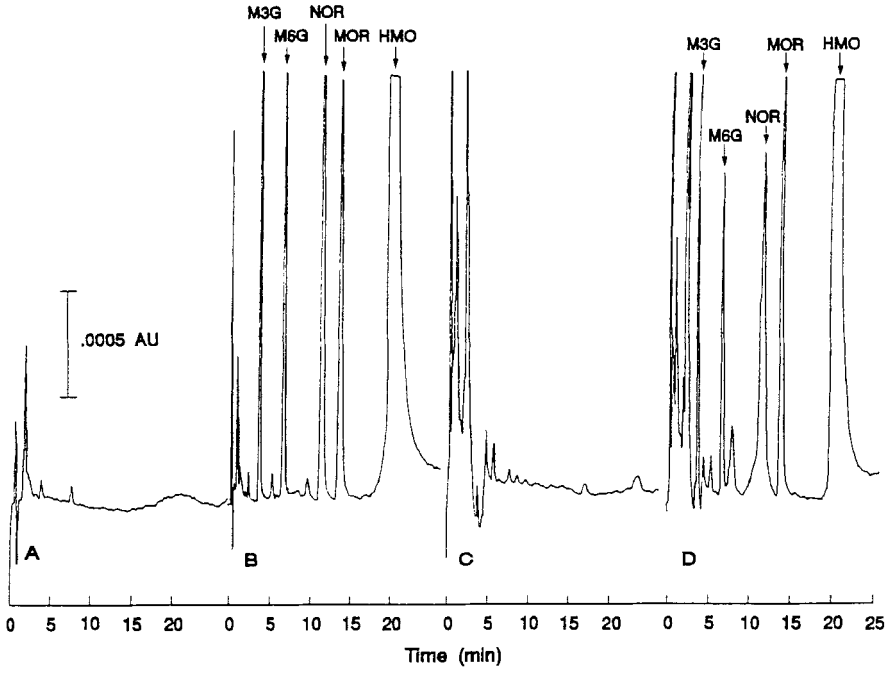

FIGURE 1. Chromatograms of (A) mobile phase; (B) mobile phase with 20 ng/100 μl injection of M6G, M3G, NOR, morphine and 225 ng/100 μl injection of hydromorphone (HMO); (C) extracted blank guinea pig plasma; and (D) extracted guinea pig plasma with 20 ng/100 μl injection of M6G, M3G, NOR and morphine, and 225 ng/100 μl injection of hydromorphone.

RESULTS

Resolution and Linearity

Well resolved peaks for morphine, M6G, M3G, NOR and hydromorphone were obtained using this method, and no interfering peaks were encountered in plasma (Figure 1) or microsomal preparations. Standard curves for all compounds were linear. Figure 2 shows typical

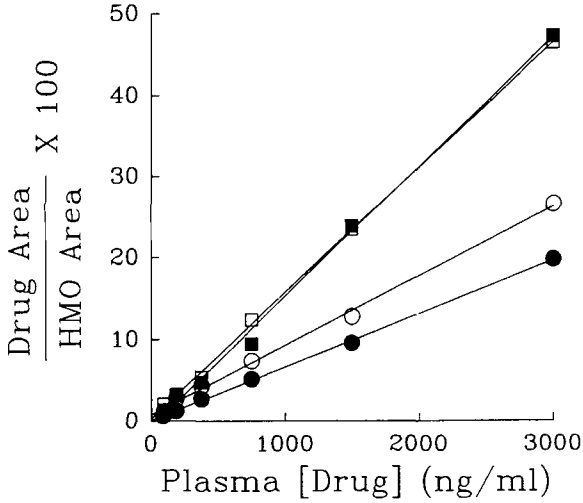

FIGURE 2. Standard curves for M6G (●), M3G (○), NOR (■) and morphine (□). An 80 μl sample of guinea pig plasma was extracted on a C-18 solid phase column, the extract dried under nitrogen and reconstituted in mobile phase. The ratios of the peak area for each drug to the internal standard area, multiplied by 100 are plotted as functions of the amount of drug in unextracted plasma. Curves are produced from least squares regression analysis. Pearson's correlation coefficients (r^2) were 0.999 for morphine and M6G, 0.997 for M3G and 0.996 for NOR.

standard curves for each compound. The r^2 values of the curves for morphine and M6G were 0.999, M3G was 0.997 and NOR was 0.996.

Sensitivity, Specificity, Recovery, Precision and Accuracy

Considering the 80 μl sample size and the dilutions inherent to the assay, the sensitivity of the assay was 23 ng/ml for M6G, M3G and NOR and 47 ng/ml for morphine for an injection volume of 100 μl. The lowest quantifiable amount for all compounds was 94 ng/ml. Other assays using

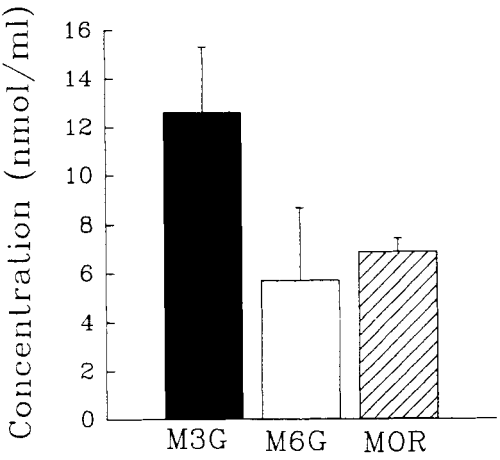

FIGURE 3. Production of active morphine metabolites in neonatal guinea pigs. Blood was collected 35-40 min after three 15-17 day old pups were injected subcutaneously with 15 mg/kg morphine. Error bars represent standard deviation.

single wavelength UVD have reported lower limits of quantitation[16,22], however, the most sensitive of these assays requires a prohibitively large sample volume (1.0 ml of plasma)[16] for use in small animals. The microassay presented here has the sensitivity required for developmental pharmacology studies in small (Figure 3) and large[14] animal models.

The assay is specific for morphine and its metabolites and is able to distinguish the natural morphine-6-β-D-glucuronide from the stereoisomer morphine-6-α-D-glucuronide. Relative retention times of related compounds are listed in Table 1.

Data on recovery, precision and accuracy were determined at a drug concentrations of 750 ng/ml, the midpoint of the standard curve. These data are presented in Table 2.

TABLE 1
Retention Times of Various Compounds Relative to Morphine[a,b].

Compound	*Relative retention time*
Morphine-3-β-D-glucuronide	0.28
Apomorphine	0.39
Morphine-6-α-D-glucuronide	0.40
Morphine-6-β-D-glucuronide	0.48
Normorphine	0.83
Dihydromorphine	0.90
Morphine-3-ethereal sulfate	0.99
Morphine	1.00
Oxymorphone	1.02
Hydromorphone[c]	1.50
Naloxone	2.29
Nalorphine	2.78

[a]20 ng of unextracted compound was injected and the chromatogram recorded for 30 min.
[b]No peaks were seen during the 30 min for amphetamine, benzoylecgonine, benzoylnorecgonine, cocaine, codeine, l-methadone, nalbuphine, norcocaine, or oxycodone.
[c]225 ng was injected.

In Vivo and In Vitro Metabolite Formation

The production of active morphine metabolites was investigated in three neonatal guinea pigs. Blood was sampled 35-40 min after a s.c. injection of 15 mg/kg morphine and reported as nmol/ml to correct for differences in the molecular weights of the compounds (Figure 3). The molecular weights of morphine and the two glucuronides are 285 and 461

TABLE 2
Recovery, Coefficient of Variation and Accuracy for Morphine, Morphine-6-β-Glucuronide, Morphine-3-β-Glucuronide, Normorphine and Hydromorphone.

Drug	*Recovery*[a] *(%)*	*Coefficient of Variation (%)*[a,b]	*Accuracy*[a] *(%)*
Morphine	100	4.9	95.6
Morphine-6-β-D-Glucuronide	92	2.0	97.0
Morphine-3-β-D-Glucuronide	85	6.3	97.2
Normorphine	84	5.4	90.0
Hydromorphone	95	5.7	-

[a]Plasma concentration of 750 ng/ml. Accuracy was defined as observed divided by expected values, multiplied by 100.
[b]n=6.

ng/nmol, respectively. This dose is the upper range used in respiratory studies in this laboratory and the time point was chosen to correspond to peak respiratory depression. Morphine represented (mean ± S.D.) 28.3 ± 7.7% of total morphine equivalents (conjugated + unconjugated drug) in plasma at the time of sampling. M6G and M3G accounted for 21.6 ± 8.1% and 50.1 ± 3.3% of total morphine equivalents, respectively. NOR was not detected in any samples.

In a separate study, the in vitro oxidative metabolism of morphine by hepatic microsomal enzymes from a pregnant guinea pig was investigated using the assay reported here. NOR production was linear over the first 20 min (r^2 = .945) and the initial rate was 0.439 nmol/min/mg of protein.

DISCUSSION

Previous assays for morphine and at least one metabolite have been reported, however the sample sizes required by these methods are too large for use with small animals. The 80 μl sample size in this microassay is one-fifth the requirement of the next smallest assay's sample volume[21]. The limit of quantitation of any assay is determined in part by the sample volume. Lower sensitivities have been reported[16,22], but with much larger sample sizes. The limit of quantitation of 94 ng/ml for morphine and metabolites reported here is the optimum obtainable with a small sample size, and represents adequate sensitivity for the drug levels seen in developmental work with small animals. The use of C-18 Bond Elut columns in conjunction with the Vac Elut manifold as reported here allows for a rapid extraction process and for the simultaneous preparation of up to ten samples. In both respects, this is an improvement over the previously reported use of Chromprep PRP-1[14] or Sep-Pak C_{18} cartridges[16,17,22,23]. Some assays[16-18] have also reported the use of a double extraction, using two solid phase cartridges in order to remove interfering peaks. The addition of a 1 ml dichloromethane wash to the current extraction removes such peaks and allows a single extraction per sample. Various volumes of the ammonium sulfate and dichloromethane washes were tested, and the method described produced minimal interfering peaks with no loss of drug. A 10 mM NaH_2PO_4 buffer, pH 2.1 with acetonitrile has been used to elute drugs from the extraction

cartridges[14,16,17,22,23], however, this resulted in low and inadequate recoveries when applied to a small sample size. The acidified 90:10 methanol:dichloromethane mixture presented here was found to improve recovery by 30-40%. A range of mixtures of methanol:dichloromethane from 100:0 to 50:50 were examined, and 90:10 gave the best recovery, with the acidification further improving upon the recovery obtained by use of solvents alone. Hydromorphone was chosen for the internal standard due to its retention time and good recovery (95%).

Related opioid compounds naloxone and nalorphine chromatograph under the conditions of this assay, however, they are lost in extraction due to the dichloromethane wash. The relative retention times of all compounds could be increased with decreasing amounts of acetonitrile in the mobile phase. Increase in the concentration of the ion pairing agent, SDS, also increased the retention time of morphine and its metabolites.

Both active glucuronide metabolites were produced in neonatal guinea pigs following morphine exposure. Further investigation of any role these compounds may play in morphine pharmacodynamics is being conducted. While NOR was not seen in the neonate, hepatic microsomes from the pregnant guinea pig are capable of rapid NOR production, and this is being characterized. Phase II (conjugation) metabolism of morphine has also been studied *in vitro* with this assay (data not shown).

In conclusion, an HPLC microassay for the simultaneous determination of morphine and its active metabolites is described. The

assay is stereospecific and separates the isomers morphine-6-α-D-glucuronide and morphine-6-β-D-glucuronide. The small sample size and sensitivity are suitable for use with *in vivo* and *in vitro* developmental pharmacology studies. The unique stereospecific aspect of this assay would also allow for a quality control application following the chemical synthesis of M6G. The method is reproducible and provides rapid extraction of multiple samples with good recovery of all compounds. The neonatal guinea pig produces both glucuronides *in vivo* while hepatic microsomes from the dam produce NOR *in vitro*.

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