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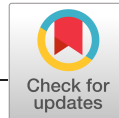
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RESEARCH ARTICLE

Development of a method for the determination of hydromorphone in plasma by LC-MS

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Abstract

A simple high-performance liquid chromatography method for the determination of hydromorphone in small volume plasma has been developed. Following solid-phase extraction using Oasis HLB cartridges, samples were separated by reverse-phase high-performance liquid chromatography on an Atlantis T3 4.6 × 150 mm column (3.0 μm) and quantified using mass spectrometry. The mobile phase was a mixture of water with 0.1% formic acid and acetonitrile with 0.1% formic acid (91:9). The standard curve ranged from 1 to 500 ng/mL. Intra- and Inter-assay variability for hydromorphone was <10%, and the average recovery was >90%. The LLOQ was 1 ng/mL. This method was successfully applied to the analysis of hydromorphone samples at this institution. This method could be useful to those investigators dealing with small sample volumes, particularly when conducting pharmacokinetic studies that require multiple sampling from the same animal.

KEYWORDS

hydromorphone, MS, pharmacokinetics, solid-phase extraction

1 | INTRODUCTION

Recognition of pain and appropriate analgesic therapy is becoming increasingly important in veterinary medicine. Opioids are a diverse group of drugs that bind to specific receptors in the brain, spinal cord, and peripheral tissues. They are used for their analgesic properties, acting on the μ -, κ - and δ -opioid receptors in the central nervous system and peripheral nervous system (Lamont & Mathews, 2007). Hydromorphone is a semi-synthetic μ -opioid receptor agonist with a potency 5–7 times that of morphine (Murray & Hagen, 2005). Extensive research studies support the clinical use of hydromorphone in pain management protocols (Kelly, Pypendop, & Christe, 2014). It is used frequently in the USA in small animal anesthesia and for pain management because of its low cost and apparent good efficacy and safety history (Pettifer & Dyson, 2000). In addition to use in mammals, hydromorphone analgesic efficacy has been validated in red-eared slider turtles (Mans, Lahner, Baker, Johnson, & Sladky, 2012). It is also an accepted alternative to morphine because it does not cause histamine release after i.v. administration (Pettifer & Dyson, 2000; Smith, Yu, Bjorling, & Waller, 2001).

Despite the rising popularity of hydromorphone in reptile medicine in the USA, reptile formularies do not contain dosing recommendations for analgesic regimens in reptiles. Doses are often adjusted based on pharmacokinetic and pharmacodynamics principles known from domestic animals (Sladky, Mader, & Divers, 2014). Pharmacokinetic data from studies indicate a large degree of variation between species. The half-life in cats, macaques, kestrels and dogs is 98.9, 142, 75 and 60 min, respectively (Wegner, Robertson, Kollias-Baker, Sams & Muir, 2004; KuKanich, Hogan, Krugner-Higby & Smith, 2008; Kelly, Pypendop & Christe, 2014; Guzman Sanchez-Migallon, KuKanich, Drazenovich, Olsen & Paul-Murphy, 2014). Clearance is 24.6, 37.7, 62.3 and 60 mL/min/kg in cats, macaques, kestrels and dogs. (Guzman Sanchez-Migallon et al., 2014; Kelly et al., 2014; KuKanich et al., 2008; Wegner et al., 2004), while the volume of distribution at steady state is 2957, 4062, 1164 and 4410 mL/kg and the area under the curve is 4079, 1993, 3508 and 7350 mL/min/kg in cats, macaques, kestrels and dogs, respectively (Guzman Sanchez-Migallon et al., 2014; Kelly et al., 2014; KuKanich et al., 2008; Wegner et al., 2004). The lack of pharmacokinetic data for analgesic agents in reptiles makes it impossible to determine appropriate doses, dosing intervals and safety, hampering the appropriate selection and

use of analgesics in clinical practice. As such, it is difficult to recommend effective and safe dosing intervals for reptiles. Owing to the large degree of variation in metabolism, pharmacokinetic parameters must be established for each species.

There have been some methods reported for the determination of hydromorphone in human plasma which involved radioimmunoassay (Lee et al., 1991), high-performance liquid chromatography using electrochemical detection (Wetzelsberger, Lucker, & Erking, 1986; Bouquillion, Freeman, & Moulin, 1992; Guedes, Papich, Rude & Rider, 2008) and high-performance liquid chromatography tandem mass spectrometry (MS) (Chen, Hanson, Jiang, & Naidong, 2002; Coles, Kushnir, Nelson, McMillin, & Urry, 2007; Dahn, Gunn, Kriger, & Terrell, 2010; Kelly et al., 2014; KuKanich et al., 2008; Musshoff, Trafkowski, Kuepper, & Madea, 2006; Naidong et al., 2000; Wegner et al., 2004). The radioimmunoassay method (Lee et al., 1991) lacks selectivity and requires the use of radioactivity, which requires a license and can be costly to dispose of, and a large volume of plasma. The electrochemical detection methods require large sample volumes, have poor recovery and are not as sensitive (Wetzelsberger et al., 1986, Bouquillion et al., 1992, Guedes et al., 2008).

Many of the LC-MS methods use large sample sizes and have more complex time-consuming extractions. The methods used in the studies by Kelly et al. (2014) and KuKanich et al. (2008) required larger sample sizes (0.5 and 1 mL) and were not as accurate or precise. The method used by Kelly et al. (2014) does have a lower LOQ (0.1 ng/mL) but they used a triple quadrupole detector and a larger sample size to obtain the value. One method (Chen et al., 2002) requires the use of a robotic liquid handler which is costly and may not be available in many laboratories. It also has poor recovery while another method (Musshoff et al., 2006) requires cannula cleaning for each sample during the extraction phase, which is time consuming. The method developed by Coles et al. (2007) is a complicated procedure that requires the number of drops per second passing through the solid-phase extraction cartridge to be controlled and monitored. In the method by Wegner et al. (2004) they used a 0.05 mL sample; however, a 30% variation in concentration was acceptable and no LOQ, recovery, accuracy or precision data was listed. The Guzman Sanchez-Migallon et al. (2014) method had a smaller sample size (0.05 mL) but used only three replicates, not five, for validation and used a different plasma than the actual sample for the validation process. The method developed by Naidong et al. (2000) used normal-phase high-performance liquid chromatography MS to improve sensitivity and peak shape; however, that type of chromatography is not commonly used in many laboratories. By optimizing chromatography conditions hydromorphone can be detected using reverse-phase chromatography.

The aim of this paper is to describe a simple, sensitive and accurate method for extracting hydromorphone from small-volume plasma samples. This method has been successfully applied to bearded dragon samples analyzed at the author's institution and should be applicable to other species. It could be useful to those investigators dealing with small sample volumes, particularly when conducting pharmacokinetic studies that require multiple sampling from the same animal.

2 | EXPERIMENTAL

2.1 | Chemicals and standards

Hydromorphone and naltrexone (Figure 1) were purchased from US Pharmacopeia (Rockville, MD, USA). Both chemicals were >99% purity. All other mass spectrometry grade chemicals and solvents were purchased from Fisher Scientific (Pittsburg, PA, USA).

Stock solutions of hydromorphone (100 µg/mL) and naltrexone (100 µg/mL), which is the internal standard, were prepared in methanol. Dilutions in methanol were prepared to produce 0.01, 0.1 and 1 µg/mL working stocks. Solutions were aliquoted into separate vials to prevent evaporation and cross-contamination. All solutions were stored at 4°C. By comparing standard areas over time it was determined that they were stable for a minimum of 6 months.

For preparation of calibration standards and quality control samples, appropriate aliquots of the working stock solutions were placed in tubes, evaporated to dryness with nitrogen and then dissolved in 100 µL of untreated lithium heparin bearded dragon plasma. The plasma was obtained from drug-free healthy animals. The final concentrations were 1, 2.5, 5, 10, 25, 50, 100, 250 and 500 ng/mL for calibration standards and 3.5, 15, 35, 75, 150 and 350 µg/mL for quality control samples. Calibration standards and control samples were treated identically to samples. Linearity was assessed by linear regression analysis. The calibration curve had a correlation coefficient of ≥ 0.99 . The acceptance criterion for each back-calculated standard and quality control concentration was 15% deviation from the nominal value except for the lower limit of quantification (LLOQ), which was set at 20%.

2.2 | Chromatographic conditions and apparatus

The chromatography system consisted of a 2695 separation module and an Acuity QDa single-quadrupole mass detector (Waters, Milford, MA, USA). Separation was achieved on an Atlantis T3 column (4.6 × 150 mm, 3 µm) preceded by a 3 µm Atlantis T3 guard column. The mobile phase was a mixture of (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid (91:9). The mixture was pumped at a starting gradient of 91% A and 9% B and held for 4 min, and then was adjusted to 85% A and 15% B over 6 min, and back to initial conditions over 3 min. The flow rate was 0.70 mL/min, and the column temperature was 30°C. The compounds were detected by positive selected ion recording. The scan rate was 2 points/s, gain 1, capillary voltage 0.6 kV, cone voltage 12, ion source temperature 150°C and probe temperature 600°C. Nitrogen was used

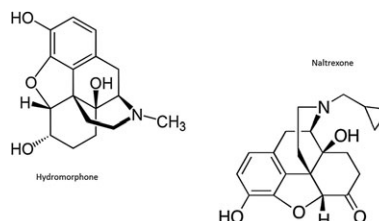


FIGURE 1 Structures for hydromorphone and naltrexone

as the nebulizing gas. Hydromorphone was detected at 286.10 m/z and naltrexone was detected at 342.41 m/z .

2.3 | Sample preparation

Hydromorphone was extracted from plasma samples using a solid-phase extraction method with Oasis HLB cartridges (Waters, Milford, MA, USA). Briefly, previously frozen plasma samples were thawed and vortexed and 100 μL was transferred to a 13 \times 100 mm glass tube then 60 μL of internal standard (0.1 $\mu\text{g}/\text{mL}$ naltrexone) was added. Three hundred microliters of water-methanol (65:35) was added and tubes were vortexed and allowed to stand for 3 min, and then centrifuged for 15 min at 1020g. The supernatant was added to a prewet Oasis 1 cm^3 cartridge (1 mL methanol, 1 mL of water) and washed with 1 mL of 3% methanol in water. Samples were eluted

with 1.0 mL of 2% formic acid in acetonitrile-methanol (40:60) and then evaporated to dryness with nitrogen gas. Samples were reconstituted in 250 μL of mobile phase and 60 μL injected into the HPLC system.

3 | RESULTS AND DISCUSSION

3.1 | Method development and optimization

We wanted to develop a simple, straightforward method that would be effective with small volume samples. Several organic solutions and mixtures were tested during optimization of the extraction procedure, including acetonitrile, methanol, trichloroacetic acid, ethyl acetate, hexane, and methylene chloride. All were found to produce

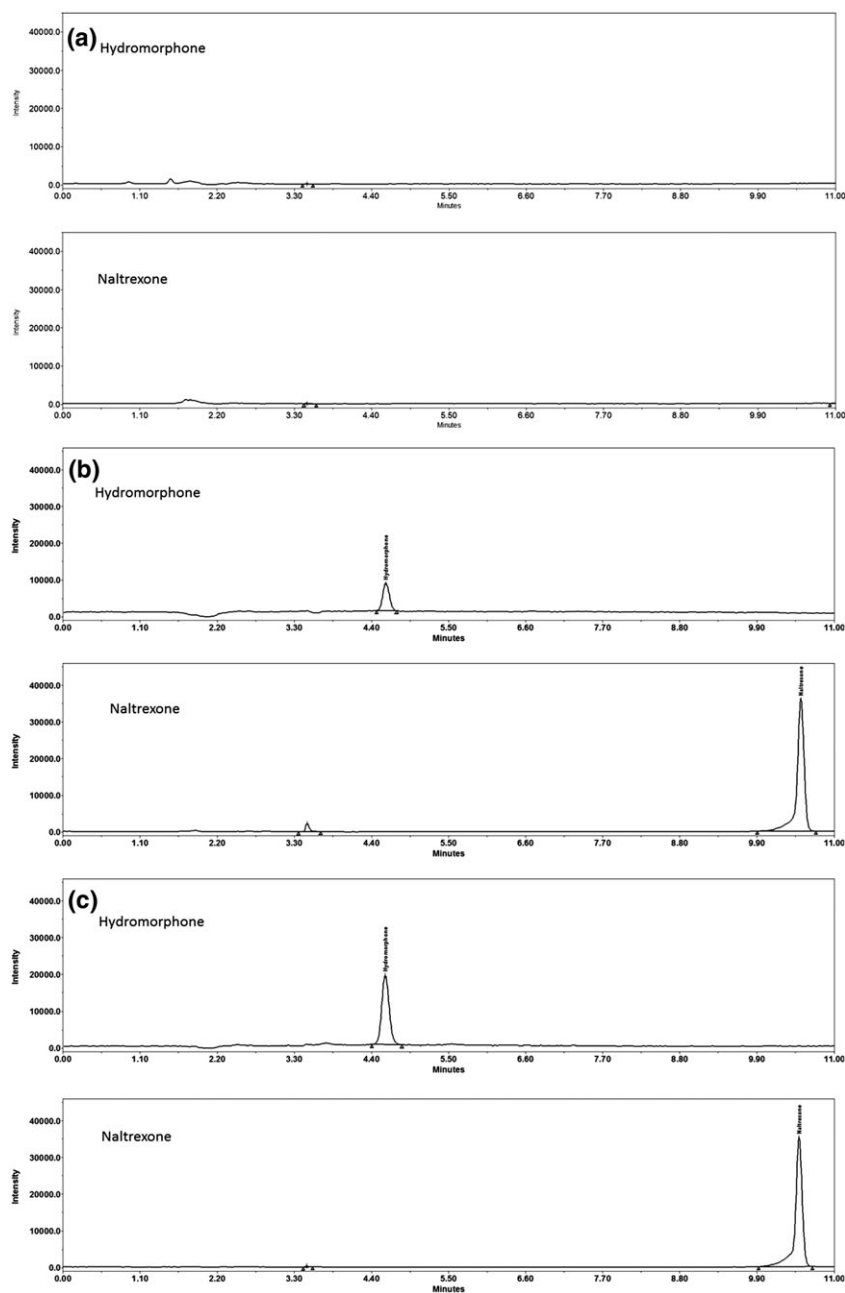


FIGURE 2 Chromatograms for hydromorphone in bearded dragons. (a) A blank bearded dragon sample; (b) a 25 ng/mL spiked plasma standard; and (c) a plasma sample from a bearded dragon 1 h after an 0.5 mg/kg subcutaneous dose of hydromorphone was administered

lower recoveries compared to the SPE technique with Oasis HLB cartridges.

During the development of the SPE technique several different cartridges were examined besides the Oasis HLB (Waters, Milford, MA, USA), including Strata-X, Strata-XA (Phenomenex, Torrance, CA, USA) and Sep pak plus (Waters, Milford, MA, USA). Use of Oasis HLB cartridges, with a hydrophilic-lipophilic balanced copolymer, enabled high recoveries for both hydromorphone and naltrexone; all others had very low recoveries or poor peak resolution. Once an appropriate cartridge was selected we also looked at various wash solution combinations, including 1, 3, 5 and 7% methanol in water. The 5 and 7% solutions caused a decrease in recovery and the 1% solution produced an eluent that when re-dissolved could not be injected into the system because of particulates. The 3% solution was found to produce the greatest recovery with the best peak resolution. The elution composition was part of a standard protocol recommended by the manufacturer which worked well for the plasma extraction. The amount was increased from 300 μ L twice to 500 μ L twice. This increased the recovery by roughly 15%.

3.2 | Method validation

3.2.1 | Selectivity

For specificity testing, untreated plasma from bearded dragons was prepared in the same manner as study samples and no endogenous components interfered with elution of the compounds of interest. Six different blank samples were used in the pre-validation process and a blank sample from each animal was included in the analysis. The elution chemicals were also checked for possible chromatogram interference. Figure 2 illustrates selected ion recording chromatograms of (a) a blank bearded dragon sample, (b) a 25 ng/mL spiked plasma standard and (c) a plasma sample from a bearded dragon 1 h after subcutaneous administration of a 0.5 mg/kg dose of hydromorphone. The retention times in plasma for hydromorphone and naltrexone were 4.60 and 10.50 min in bearded dragons, respectively. Drugs that could be used in combination with hydromorphone in treatment protocols include meloxicam, dexmedetomidine, ketamine and/or midazolam. These drugs did not interfere with the analysis of hydromorphone.

3.2.2 | Linearity, accuracy and precision

The plasma peak ratio (area of hydromorphone divided by the internal standard area) vs the concentration was plotted and produced a linear curve for the concentration range used (1–500 ng/mL) with a correlation coefficient of 0.9992. A typical equation for the calibration curve was $y = 0.0081x + 0.0020$ (Table 1) where y represents the peak area ratio of hydromorphone to internal standard and x represents the plasma concentration of hydromorphone in nanograms per milliliter. Intra- and inter-day assay coefficients of variation (CV) for plasma spiked with specific concentrations of hydromorphone ranged from 1.2% to 9.3%. The precision was found to be well below the set $\pm 15\%$ for all quality control samples as shown in Tables 1 and 2.

TABLE 1 Intra-assay accuracy, precision and assay linearity for hydromorphone in plasma

Intra-assay variability (n = 5)			
Concentration added (ng/mL)	Concentration measured (ng/mL) (mean \pm SD)	Accuracy (%)	CV (%)
3.5	3.5 \pm 0.21	100	5.9
15	15 \pm 0.91	100	6.1
35	33 \pm 3.08	94	9.2
75	72 \pm 3.56	96	5.0
150	157 \pm 9.13	105	5.8
350	354 \pm 4.1	101	1.2
Assay linearity (n = 5)			
	Mean \pm SD	CV (%)	
y-Intercept	0.0020 \pm 0.0001	5.0	
Slope	0.0081 \pm 0.0005	6.1	
r ²	0.9992 \pm 0.0002	0.02	

SD, Standard deviation; n, number of samples; CV, coefficient of variation.

TABLE 2 Inter-assay variability for hydromorphone in plasma and (n = 5)

Concentration added (ng/mL)	Concentration measured (ng/mL) (mean \pm SD)	Accuracy (%)	CV (%)
3.5	3.4 \pm 0.17	99	5.2
15	15 \pm 1.08	100	7.1
35	36 \pm 1.41	103	4.0
75	74 \pm 6.92	99	9.3
150	152 \pm 6.49	101	4.3
350	353 \pm 10.38	101	2.9

SD, Standard deviation; n, number of days; CV, coefficient of variation.

3.2.3 | Recovery, LLOQ

The recovery (Table 3) of hydromorphone from spiked plasma was determined by comparing the extracted areas with the directly injected analyte areas and ranged from 88 to 99%. The relative standard deviation was $<10\%$ for all values. The recovery of the internal standard was 92% and its relative standard deviation was 2.1%. The use of naltrexone as an internal standard corrects for the intra- and inter-day assay variability in the assay. The LLOQ was 1 ng/mL, which represents a peak ~ 5 times baseline noise. The LLOQ is more than adequate for use in pharmacokinetic studies. If a lower LLOQ is

TABLE 3 Recovery for hydromorphone in plasma and (n = 5)

Concentration added (ng/mL)	Recovery (%)
3.5	99
15	90
35	90
75	88
150	93
350	91

n, Number of days.

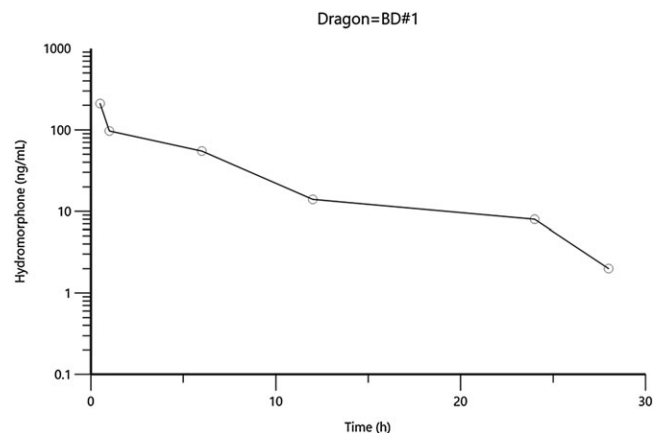


FIGURE 3 Concentration–time profile of hydromorphone in a bearded dragon after a 0.5 mg/kg subcutaneous dose

needed, the sample size could be increased. The lower limit of detection was 0.5 ng/mL, which represents a peak ~3 times baseline noise.

3.3 | Stability

Testing of the short-term stability of the quality control standards indicated that there was a 3% loss of hydromorphone after 24 h in the autosampler and 14% drug loss after 24 h in the refrigerator at 4°C. Therefore, if there were a power or equipment failure, samples could be reanalyzed, especially if they were not refrigerated. Samples in our studies were thawed once and analyzed; however, there was no loss of drug after three freeze–thaw cycles.

4 | CONCLUSION

In conclusion, this analytical procedure was validated in terms of recovery, linearity, LLOQ, precision and accuracy. The limit of quantification and recovery are more than adequate for use in pharmacokinetic studies. Our results indicate that this HPLC procedure is a reproducible method that provides consistent quantification of hydromorphone in plasma. The addition of naltrexone as an internal standard also allows for the correction of intra- and inter-assay variability. This method has been used successfully to determine hydromorphone concentrations in plasma samples at this institution. Figure 3 is a representative concentration–time profile from a hydromorphone pharmacokinetic study conducted in bearded dragons after receiving a 0.5 mg/kg subcutaneous dose of hydromorphone. This method should be applicable to other species.

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