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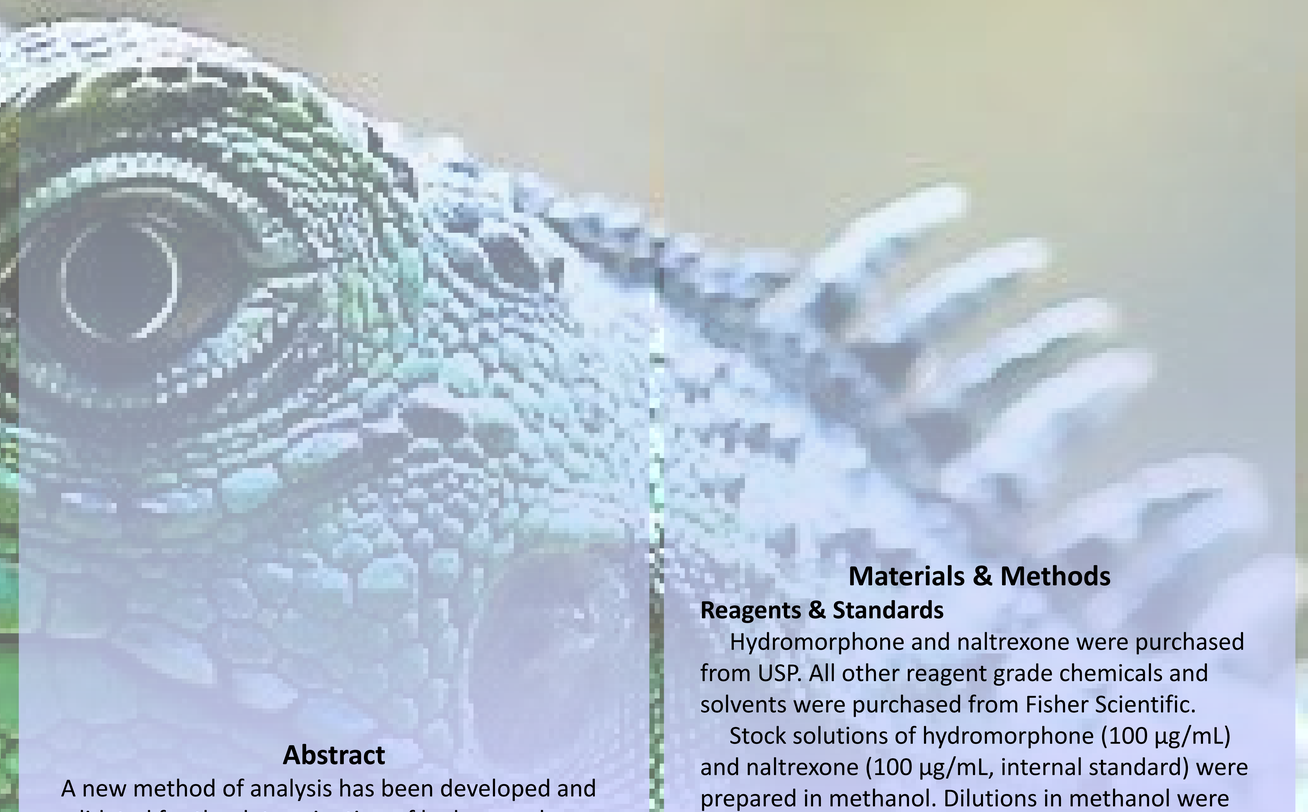


Abstract

A new method of analysis has been developed and validated for the determination of hydromorphone in bearded dragon plasma. Following SPE, samples were separated by reversed-phase HPLC on an Atlantis T3 column (4.6 x 150 mm, 3 µm) and detected by MS. The mobile phase was a mixture of water with 0.1% formic acid, and acetonitrile with 0.1% formic acid (91:9), with a flow rate of 0.7 mL/min. The procedure produced a linear curve over the concentration range of 1 to 500 ng/mL with an LOQ of 1 ng/mL and LOD of 0.5 ng/mL. Intra- and inter-assay variability ranged from 3.9% - 9.3% and the average recovery for hydromorphone was 91%. The average recovery for naltrexone, which was the internal standard, was 90%. This method has been developed in bearded dragon plasma and should be applicable to other species, it should also be useful to those investigators dealing with small volumes, particularly when conducting PK studies that require multiple sampling from the same animal.

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 CNS and peripheral nervous system. Hydromorphone is a semi-synthetic µ-opioid receptor agonist with a potency of five to seven times that of morphine. It is used frequently in small animal anesthesia and for pain management because of its low cost and apparent efficacy and safety history. In addition, hydromorphone is an accepted alternative to morphine because it does not cause histamine release after IV administration.

Materials & Methods

Reagents & Standards

Hydromorphone and naltrexone were purchased from USP. All other reagent grade chemicals and solvents were purchased from Fisher Scientific.

Stock solutions of hydromorphone (100 µg/mL) and naltrexone (100 µg/mL, internal standard) were prepared in methanol. Dilutions in methanol were prepared to produce 0.01, 0.1, and 1 µg/mL working stocks. All solutions were stored at -20˚C. By comparing standard areas over time it was determined that they were stable for six months.

For preparation of calibration standards and quality control (QC) 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 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 QC samples.

Linearity was assessed by linear regression analysis. The calibration curve had a correlation coefficient of 0.99 or better. The acceptance criterion for each back-calculated standard and QC concentration was 15% deviation from the nominal value except the lower limit of quantification (LOQ) which was set at 20%.

Chromatographic conditions and apparatus

The chromatography system consisted of a 2695 separation module and an Acuity QDa single-quadrupole mass detector (Waters). The compounds were separated on an Atlantis T3 (4.6 x 150 mm, 3 µm) column 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 minutes 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 (SIR).

Chromatographic conditions and apparatus

The scan rate was 2 pts/s, gain 1, capillary voltage 0.6 kV, cone voltage 12, ion source temperature 150˚C and probe temperature 600˚C. Nitrogen was used as the nebulizing gas. Hydromorphone was detected at 286.10 m/z and naltrexone was detected at 342.41 m/z.

Sample preparation

Hydromorphone was extracted from plasma samples using a solid phase extraction method with Oasis HLB 1 cc cartridges (Waters). Briefly, previously frozen plasma samples were thawed and vortexed and 100 µl was transferred to a 13 x 100 mm glass tube then 60 µl of internal standard (0.1 µg/ml naltrexone) added. Three hundred microliters of water:methanol (65:35) was added and tubes were vortexed and allowed to stand for 3 minutes, and then centrifuged for 15 min at 1020 x g. The supernatant was added to a prewet Oasis cartridge (1 mL methanol, 1 mL of water) and washed with 1 mL of 3% methanol in water. Samples were eluted with 1 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.

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 ± S.D.)	Accuracy (%)	C.V. (%)
3.5	3.5 ± 0.21	100	5.9
15	15 ± 0.91	100	6.1
35	33 ± 3.08	94	9.2
75	72 ± 3.56	96	5.0
150	157 ± 9.13	105	5.8
350	354 ± 4.1	101	1.2

Assay linearity (n = 5)

	Mean ± S.D.	C.V. (%)
Y-Intercept	0.0020 ± 0.0001	5.0
Slope	0.0081 ± 0.0005	6.1
r^2	0.9992 ± 0.0002	0.02

S.D.: standard deviation; n: number of samples; CV: coefficient of variation

Table 2. Inter-assay variability and recovery for hydromorphone in plasma and recovery (n = 5)

Concentration added (ng/mL)	Concentration measured (ng/mL) (mean ± S.D.)	Accuracy (%)	C.V. (%)	Recovery (%)
3.5	3.4 ± 0.17	39	5.2	100
15	15 ± 1.08	100	7.1	90
35	36 ± 1.41	103	4.0	88
75	74 ± 6.92	99	9.3	88
150	152 ± 6.49	101	4.3	93
350	353 ± 10.38	101	2.9	91

SD: standard deviation; n: number of days; CV: coefficient of variation.

Figure 1. Chromatograms of hydromorphone (A) 25ng/mL spiked plasma standard, (B) bearded dragon plasma sample 1 h after SC administration, (C) blank bearded dragon sample.

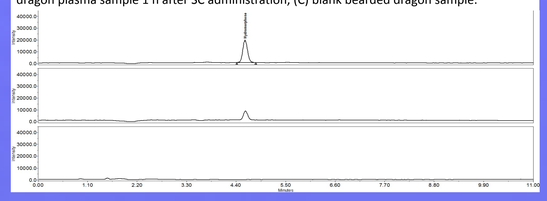


Figure 1. Chromatograms of Naltrexone (I.S.) (A) 25ng/mL spiked plasma sample, (B) bearded dragon plasma sample 1 h after SC administration, (C) blank bearded dragon sample.

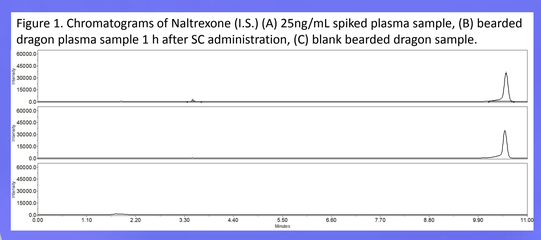
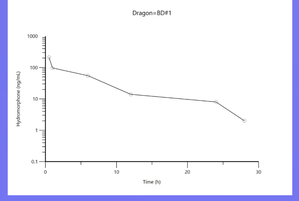


Figure 2. Concentration time profile of hydromorphone in a bearded dragon after a 0.5 mg/kg subcutaneous dose.



Results & Discussion

Blank plasma samples were prepared in the same manner as study samples and no endogenous plasma components interfered with the elution of the peaks of interest. Fig. 1 shows chromatograms of a (A) 25ng/mL spiked plasma standard, (B) a bearded dragon plasma sample 1 h after SC administration and (C) blank bearded dragon plasma. Retention times were 4.66 min for hydromorphone and 10.57 min for naltrexone. For specificity testing six different blank plasma samples were used in the pre-validation process.

The plasma peak ratio (area of hydromorphone divide by the IS area) versus the concentration was plotted and produced a linear curve, over the concentration range of 1 – 500 ng/ml, with resulting correlation coefficients of >0.999. A typical equation for the calibration curve was y = 0.0081x + 0.0020, where y represents the peak area ratios of hydromorphone to internal standard and x represents the plasma concentration of hydromorphone in ng/mL. Intra and inter-assay RSD for plasma spiked with specific concentrations of hydromorphone ranged from 1.2 to 9.3% (Tables 1 and 2). The precision was below the set ± 15% for all quality control samples.

The recovery (Table 2) for hydromorphone from spiked plasma was determined by comparing extracted areas with directly injected analyte areas and ranged from 88% to 100%. The relative standard deviation was less than 10% for all values. The recovery of naltrexone was 90% and its relative standard deviation was 2.0%. The LOQ was 1 ng/ml and represents a peak approximately five times baseline noise.

Conclusions

In conclusion, this analytical procedure was validated in terms of recovery, linearity, LOQ, precision, and accuracy. The LOQ 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 2 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.