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Rapid quantification of the non-competitive NMDA antagonist MK-801 in canine cerebrospinal fluid and plasma by capillary gas chromatography-nitrogen phosphorus detection

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Abstract

A facile and sensitive method utilizing solid-phase cartridge extraction and capillary gas chromatography (GC) with nitrogen phosphorus detection was validated for the determination of MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclo-hepten-5,10-imine maleate], a non-competitive NMDA receptor antagonist, in dog cerebrospinal fluid (CSF) and plasma. Clonidine hydrochloride was used as the internal standard (ISTD), after evaluation of several ISTD candidates. Separations were performed with an intermediate polarity fused silica capillary column, yielding typical retention times of 3.20 min for MK-801 and 4.90 min for ISTD. Plasma and CSF samples were extracted with100 mg Bond Elut C_{18} TCA© cartridges to yield methanolic eluates that were evaporatively enriched before reconstitution in anhydrous ethanol prior to injection. The standard curve was validated from 1 to 100 000 ng/ml for CSF, and from 0.1 to 1000 ng/ml for plasma. Chromatograms from naive plasma and CSF exhibited no endogenous interfering peaks. The efficiency of extraction recovery was >94%, and the intra-assay and inter-assay precision was within 9% relative standard deviation (%R.S.D.) for both fluids. MK-801 and ISTD were stable in the injection solvent at 22 °C for at least 48 h. The assay was applied to the toxocologic study of intrathecal MK-801 administration in the dog.

Keywords: Dizocilpine; MK-801; Cerebrospinal fluid; Plasma; Gas chromatography; NPD; NMDA antagonist; Canine

1. Introduction

MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclo-hepten-5,10-imine maleate, M.W. 337.37] is a highly potent and selective non-competitive *N*-methyl-D-aspartate-type (NMDA) receptor antagonist that acts at the NMDA receptor-operated ion channel as an open channel

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blocker [1]. Originally developed as an anticonvulsant [2], MK-801 has been limited in it's clinical application by negative systemic side effects [3,4]. Despite this clinical limitation, MK-801 has proven valuable to the study of NMDA receptor-mediated spinal pain transmission, relevant to the consideration of central sensitization and tonic pain [5–7].

Continuing research into the pharmacology and toxicology of NMDA receptor-mediated spinal pathways in a large animal model (dog) necessitated a rapid, economical, and sensitive assay for the quantification of MK-801 in cerebrospinal fluid (CSF) and plasma.

A limited number of studies have described quantitative assays for MK-801 in biological samples. Capillary gas chromatography (GC) was adapted based on it's potential for speedy analyses with high sensitivity and specificity, and limitations in other technologies. Radio-immunoassay was not selected due to the cost of obtaining antibodies to MK-801, safety issues associated in handling iodine-125 radioisotope, and specificity limitations inherent to polyclonal antibody-based immunoassay [8–11]. Sufficiently sensitive HPLC methods were rejected due to their longer chromatographic run times, or use of time consuming liquid–liquid extraction with hazardous chlorinated organic solvents [12,13]. Previously reported gas chromatographic methods for MK-801 analysis either lacked sensitivity [2,7] or required the use of a costly mass selective detector [13,14]. We describe a facile, rapid, and sensitive quantitative assay for MK-801 in CSF and plasma, using conventional capillary gas chromatographic instrumentation after solid phase sample extraction, in support of pharmacologic studies with MK-801 in the dog.

2. Methods

MK-801 (hydrogen maleate, M-107) was obtained from Research Biochemicals International (Natick, MA, USA) (see Fig. 1). Internal standard (ISTD) [2-(2,6-dichloroaniline)-2-imidazoline] (= clonidine) as hydrochloride (C-7897) was purchased from Sigma Chemical (St. Louis, MO, USA), as were the alternative ISTDs amitriptylene (A-8404), ketamine (K-2753), and the extraction reagents diethylamine (D-0806) and potassium bicarbonate (P-5833). Mass spectrometry grade methanol and ethanol was obtained from Burdick and Jackson (Muskeson, MI, USA). Water was purified with a Barnsted Nanopure System (Barnsted|Thermolyne, Dubuque, IA, USA), using institutional de-ionized water as source material. HPLC grade acetonitrile was procured from Fisher Scientific (Tustin, CA, USA). Bond Elut C_{18} TCA© cartridges (100 mg) were obtained from Varian (Walnut Creek, CA, USA). The alternative ISTDs dibenzosuberone (D10, 498-1) and dibenzosuberane (D10, 495-7) were purchased from Aldrich Chemical (Milwaukee, WI, USA), and purified by thin layer chromatography to purity $>99\%$ prior to use.

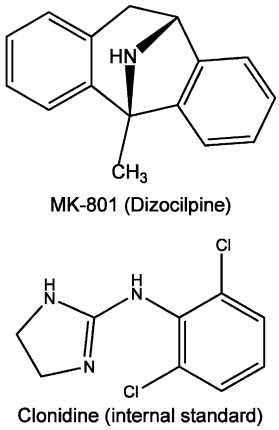

Fig. 1. Structural representation of the analyte, MK-801, $C_{16}H_{15}N$, M.W. = 221.30 and ISTD (clonidine), $C_9H_9Cl_2N_3$, M.W. = 230.10.

2.1. Gas chromatography conditions

GC was performed on a Supelco SPB-35 fused silica capillary column (30 m × 0.32 mm × 0.25 μm film thickness) (Supelco Park, PA, USA), fitted with a capillary inlet liner (4 mm i.d.) which contained a small amount of de-activated glass wool. An SPB-35 column was employed because of its ability to serve in the gas chromatographic analysis of anesthetic pharmaceuticals spanning a broad range of polarities and molecular weights,

without the need to change columns. Other columns were not evaluated. An Agilent 5890 Series II gas chromatograph, equipped with an Agilent 7673 automatic injector, housed the above column. Purified helium served as carrier, purge, and detector make up gas. Inlet pressure was maintained electronically at 20 psi, and inlet purge was initiated 0.5 min after injection, at a flow rate of 0.5 ml/min. Oven temperature was isothermal at 230 °C, injector temperature 295 °C, and the nitrogen–phosphorus detector temperature was held at 285 °C. Chromatographic parameters were optimized with pooled naive CSF or plasma extracts, containing MK-801 and ISTDs, to yield the minimal run time needed to elute MK-801 and ISTD, without interference from irrelevant peaks associated with the injection front. Agilent CHEMSTATION software (ver. A.03.11) controlled the above instrumentation and performed the peak area based quantitative calculations, after automated compensation for ISTD recovery and detector response differences. Injections (1 μl) were made in splitless mode. Autosampler tray temperature was maintained at 22 ± 1.0 °C by a recirculating water bath.

2.2. Standard solutions

Stock solutions of MK-801 and ISTD were prepared in ethanol (1000 μg/ml) and stored at −70 °C in high-density polyethylene cryovials. Working solutions were prepared daily at concentrations of 100 μg/ml with sterile saline and were used to spike samples prior to extraction. Gilford Pipetman Models P-20, P-200, and P-1000, calibrated weekly, were used for standard solution preparation, as well as for all sample manipulations.

2.3. Standard curve and quality control samples

Serial dilutions were employed to obtain final concentrations of 1–100 000 ng/ml of MK-801 in naive CSF, and 0.1–1000 ng/ml in naive plasma. These spiked samples, containing five (plasma) or six (CSF) different concentrations (0.1, 1, 10, 100, 1000, 10 000, 100 000 ng/ml), were used to construct standard curves. Quality control (QC) samples of pooled naive CSF or plasma ($n = 10$ animals) were prepared from independent stock solutions to contain concentrations of MK-801 representative of the standard curve range, and were stored at −70 °C. ISTD was added to calibration standards and QC samples immediately prior to extraction (see below). Carry-over was monitored by injection sequence randomization for calibration standards.

2.4. Sample preparation

MK-801 and ISTD were isolated from matrix by a solid phase extraction method originally designed for optimal recovery of tricyclic antidepressants from plasma [15]. To a 50 μl aliquot of CSF standard, blank, QC, or study sample in a clean 0.5 ml high density polyethylene micro centrifuge tube, 5 μl of ISTD solution was added, followed by 50 μl water, and the combined solution was mixed for 5 s. The resultant concentration of ISTD added was 10 ng/ml for plasma, 100 ng/ml for CSF. Disposable 10 μl capacity GELoader tips (Radiometer, Copenhagen, Denmark) were used for ISTD delivery, to obtain the highest possible accuracy and precision. This solution was percolated through a 100 mg Bond Elut C_{18} TCA© cartridge, previously conditioned with 0.5 ml 0.6% diethylamine in methanol and 0.5 ml 1% potassium bicarbonate in water:acetonitrile (9:1). The cartridge was then twice rinsed with 0.25 ml water:acetonitrile (8:2). Analytes were eluted with 600 μl of 0.6% diethylamine in methanol, and the eluate desiccated to dryness at 37 °C under a stream of anhydrous nitrogen. Dried eluate was reconstituted with 5 μl ethanol prior to injection. Plasma was prepared as above, using 200 μl and 5 μl sample and eluate reconstitution volumes, respectively.

2.5. Assay calibration

Calibration curves were produced by plotting peak area ratio of the analyte to the ISTD against the analyte's concentration. The linear regression was fitted to the concentration range 1–100 000 ng/ml for CSF, and 0.1–1000 ng/ml for plasma. The amount of MK-801 in samples was calculated

by the following formula: $[\text{MK-801}]_{\text{sample}} = (\text{peak area}_{\text{sample}} \times \text{MK-801 peak response factor})/(\text{peak area}_{\text{ISTD}} \times \text{ISTD peak response factor})$ X, where peak response factor = ng/peak area as derived from calibration curves.

2.6. Assay accuracy and precision

Accuracy and precision were determined by assaying QC samples (1, 100, and 10 000 ng/ml for CSF; 0.1, 10, and 100 ng/ml for plasma) in ten replicates on 6 different days. The inter-assay precision was evaluated by one-way ANOVA. Inter-assay precision, expressed as percentage RSD, was defined for each of the concentrations as:

$$\%\text{RSD} = \frac{100[(\text{TMS} - \text{EMS})/N]^{0.5}}{\text{GM}},$$

where TMS = treatment mean square, EMS = error mean square, and GM = grand mean are taken from ANOVA

2.7. Limit of quantitation (LOQ)

Pooled naive CSF or plasma ($n = 10$ animals) was analyzed simultaneously with aliquots from this same pool that had been spiked with MK-801 to a concentration of 1 or 0.1 ng/ml, for CSF and plasma, respectively. Assay accuracy at the LOQ was calculated as the percentage deviation (%DEV) for the mean observed concentration from the nominal concentration for ten aliquots. Assay precision was expressed as the relative standard deviation (%R.S.D.) of the observed concentration in the ten aliquots.

2.8. Internal standard suitability

Assay accuracy was determined for each of the candidate ISTDs by assaying groups of QC samples (1, 100, and 10 000 ng/ml for CSF; 0.1, 10, and 100 ng/ml for plasma, $n = 6$), with a different ISTD used for each group.

2.9. Extraction efficiency

Three sets of standards, within the concentration range of 1–100 000 ng/ml for CSF and 0.1–1000 ng/ml for plasma were prepared in both naive matrix and in ethanol, respectively. CSF and plasma standards were extracted and chromatographed as previously described. Standards in ethanol were injected without extraction. Extraction recovery was calculated by the following equation:

$$\%\text{Recovery} = \frac{\text{peak area found CSF (or plasma)}}{\text{peak area of unextracted ethanolic (nominal) standard}} \times 100$$

2.10. Stability

Stability of MK-801 and ISTD in injection solvent was determined by periodically injecting replicate preparations of extracted samples at 0, 12, 24, and 48 h. Peak areas obtained at the 0 h were used as reference in calculating the relative ratios for each analyte at the different time points.

3. Results and discussion

3.1. Internal standard suitability

Accuracy data for QC samples at three different concentrations of MK 801 in CSF and plasma, derived from the use of one of the five ISTD candidates are presented in Table 1. For both matrices the use of clonidine as ISTD produced the lowest %DEV values. ISTD candidates were chosen on the basis of their similarity to MK-801 in terms of molecular weight, molecular volume, polarity, and aqueous dissociation constants, with secondary consideration given to availability and cost. It is unknown exactly why clonidine produced the best accuracy. It is perhaps no coincidence that the MK-801/ISTD combination with the greatest similarity in molecular weights produced the best accuracy data.

Table 1
ISTD suitability for MK-801 quantification in canine matrices

Accuracy (%DEV)						
Matrix	Nominal, (ng/ml)	Clon[a]	AMT[b]	KET[c]	DBA[d]	DBO[e]
Plasma	0.1	9.38	−9.34	−9.14	18.79	20.32
	10	5.35	−8.72	−8.63	17.31	16.50
	100	4.72	−5.74	−6.58	11.25	9.96
CSF	1	2.03	−11.44	−8.10	18.95	18.09
	100	1.65	−10.62	−7.77	17.91	11.21
	1000	0.84	−5.80	−6.57	14.40	9.65

[a] Clon, clonidine.
[b] AMT, amitriptylene.
[c] KET, ketamine.
[d] DBA, dibenzosuberane.
[e] DBO, dibenzosuberone.

3.2. Specificity

Chromatograms of CSF and plasma obtained from naive and post-dose animals are presented in Figs. 2 and 3. Post-dose chromatograms in these two figures illustrate the detection of MK-801 at concentrations near the LOQ. No significant interfering peaks were detected at the retention times of the analytes in naive or post-dose samples, despite the expected presence of slightly more baseline noise in plasma extracts. The appearance of slightly more baseline noise in plasma extracts did not compromise overall accuracy and precision for plasma assay, as presented in Table 2. Nominal retention times for MK-801 and ISTD were 3.20 and 4.90 min, respectively.

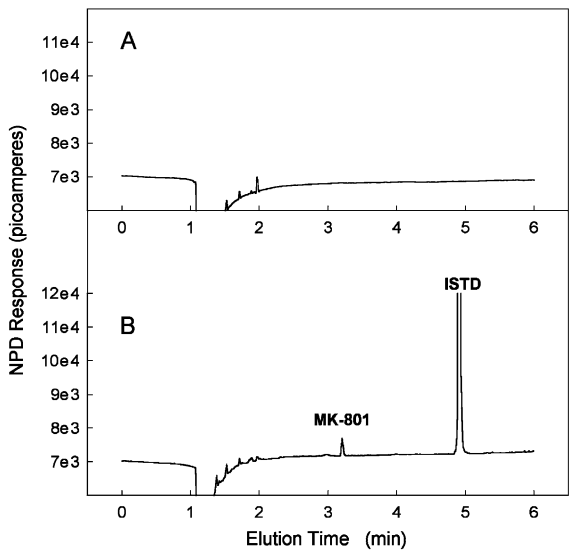

Fig. 2. Capillary gas chromatograms obtained from dog CSF, (A) pre-dose and (B) 24 h after bolus intrathecal injection of MK-801, ISTD = internal standard deviation.

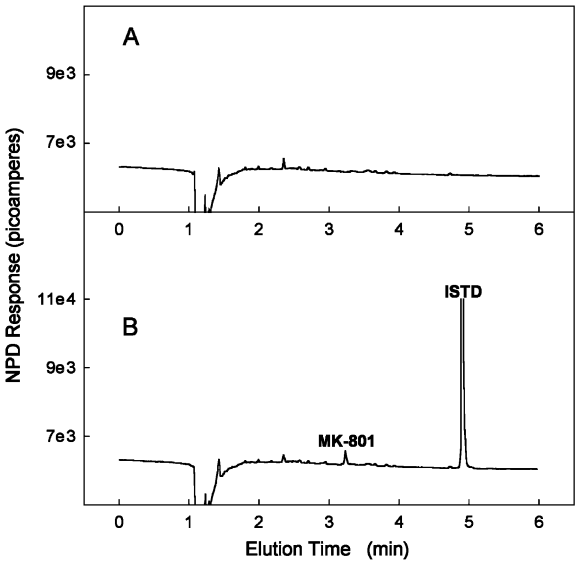

Fig. 3. Capillary gas chromatograms obtained from dog plasma, (A) pre-dose and (B) 6 h after bolus intrathecal injection of MK-801, ISTD = internal standard.

Table 2
With- and between-run accuracy and precision for MK-801 in canine matrices

	Nominal, (ng/ml)	Found, (ng/ml)	Accuracy (%DEV)	Precision (%R.S.D.)	
				Within run	Between run
Plasma	0.1	0.109	9.38	7.01	9.42
	10	10.635	5.35	4.99	5.39
	100	104.72	4.72	0.56	1.51
CSF	1	1.020	2.03	6.43	7.44
	100	101.65	1.65	2.97	4.09
	10 000	10 049.2	0.492	0.31	1.15

3.3. Linearity

Linear regression of the peak area ratios versus standard concentrations revealed that peak area ratios were linear over the concentration range of 0.1–1000 ng/ml for plasma and 1–100 000 ng/ml for CSF (data not shown). The $\%Y$ intercept for plasma and CSF were 0.013 and 0.017, respectively. The sum of residuals for plasma and CSF were 0.206 and −93.890, respectively. Values for r^2 ($r^2 \geq 0.965$ and 0.991, for plasma and CSF, respectively) and slope consistency (0.00494 ± 0.004 and 0.00345 ± 0.003, for plasma and CSF, respectively) demonstrated that standard curves were reliable for the studied concentration ranges. Analysis of co-variance for like concentrations revealed no significant difference between the plasma and CSF regression curves.

3.4. Limit of quantitation (LOQ)

The predicted mean concentration in LOQ samples for MK-801 was 0.1 and 1.0 ng/ml, in plasma and CSF, respectively. These values deviated less than $\pm 9.2\%$ from nominal values. Precision estimates for the LOQ samples were 7.0 and 6.4%R.S.D. for plasma and CSF, respectively. The LOQs for MK-801 in canine plasma and CSF were, therefore, established at 0.1 and 1.0 ng/ml, respectively. Randomization of calibration standard injections demonstrated an absence of carry-over effect (data not shown).

The reported LOQ was lower for plasma than for CSF not because of analytical parameter differences, but because measurement of MK-801 at concentrations below 1 ng/ml were not performed. Concentrations of MK-801 in CSF below 10 ng/ml were not expected in CSF, thus there was no need to validate measurement below 1 ng/ml. Given the comparative noise level of CSF and plasma chromatographic baselines (Figs. 2 and 3), one would anticipate that the absolute LOQ for CSF would be lower than that for plasma, were further experimentation to be performed.

3.5. Intra- and inter-assay accuracy and precision

Table 2 presents intra- and inter-assay accuracy and precision data for QC samples at three different concentrations of MK-801, for both plasma and CSF.

Intra- and inter-day precision values (%R.S.D.) for MK-801 were $\leq 9.4\%$. The accuracy (%DEV) for all concentrations in either matrix deviated by $\leq \pm 9.2\%$ from the corresponding nominal concentrations.

3.6. Extraction recovery

Results of the comparison of chromatographic response for unextracted ethanolic standards versus plasma or CSF spiked with MK-801 standards indicated that extraction of MK-801 was greater than 94% for all concentrations tested (Table 3).

3.7. Stability

Absolute peak area at three concentration levels of MK-801 and ISTD were found to be within $\pm$ 5.4%DEV from the corresponding peak areas at

Table 3
Extraction recovery efficiency for MK-801 ($n = 10$)

Matrix	[Nominal, ng/ml]	Peak area$_{EtOH}$	Peak area$_{Sample}$	Recovery (%diff)[a]	%R.S.D.[b]
Plasma	0.1	105	98	94	7.4
	10	10 190	9548	94	4.8
	100	1016 800	969 000	95	0.5
CSF	1	1026	1007	98	5.9
	100	1018 990	996 570	98	3.3
	1000	10179 000	10046 700	99	0.3

[a] Recovery (%diff), (peak area found from sample/peak area of unextracted ethanolic standard) × 100.
[b] %R.S.D., percent relative standard deviation.

time zero during the course of this 48 h stability experiment (data not shown).

3.8. Application

The method was applied to the measurement of cerebrospinal and plasma levels of MK-801 in dogs ($n = 3$). Following the intrathecal administration of a single 0.1 mg/kg dose of MK-801 with a single needle lumbar puncture, CSF samples were drawn at timed intervals via an indwelling (lumbar) intrathecal catheter. Blood samples for plasma harvest were simultaneously taken by cephalic vein puncture. The mean (±S.D.) lumbar CSF concentration versus time profile for MK-801 is depicted in Fig. 4. Intrathecally administered MK-801 in dogs exhibited a pharmacokinetic profile similar to that observed for lipophilic drugs in other species, consistent with a mono-exponential model of distribution and elimination [16,17]. A fit performed using iteratively re-weighted least squares analysis produced the following model equation:

$$\text{Concentration}_{(t)} = 971.6e^{-0.319t}$$

where t is the time in minutes, and concentration units are μg/ml. The elimination half-life for MK-801 from the cerebrospinal space in the dog model using the above equation was 7.2 min. Clearance and volume of distribution could be calculated from the above, however, these concepts are of limited utility for intrathecal drug delivery. For all time points, plasma concentrations were less than 3 ng/ml, therefore, providing little pharmacokinetic information (data not shown). The very low plasma levels of MK-801 observed were expected since the single intrathecal dose of MK-801 was very low.

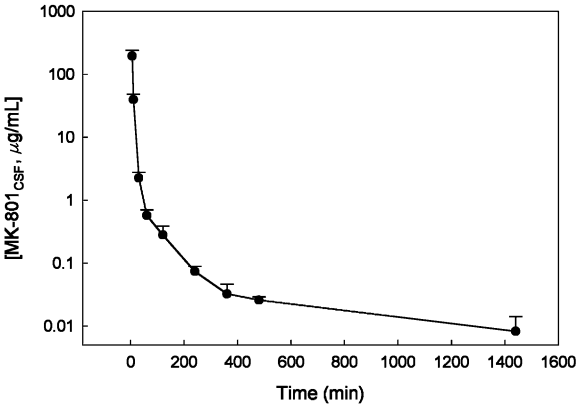

Fig. 4. Mean (±S.D.) CSF concentration versus time profile of MK-801 in dogs ($n = 3$) following a single 0.1 mg/kg intrathecal dose administration.

4. Conclusions

A capillary gas chromatographic method with thermoionic (nitrogen–phosphorus) detection was validated for determination of the NMDA antagonist, MK-801, in dog plasma and CSF. The procedure was shown to be sensitive, selective, accurate and precise. The reported method offers technological advantages, as compared with previous methods, including a rapid and simple extraction regime, short analysis times, and minimal sample volume requirements. Further, the technique is performed with widely available

supplies and relatively simple bench-top gas chromatographic instrumentation. Given the greater cost of mass selective detector-based systems, whether coupled to HPLC or GC separation modes, the GC-NPD method presented here offered a simpler, more economical alternative for the quantification needs of intrathecal MK-801 pharmacology or toxicology in the canine animal model.

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