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Short communication

Measurement of resiniferatoxin in cerebrospinal fluid by high-performance liquid chromatography

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Abstract

A sensitive and simple high-performance liquid chromatographic (HPLC) assay was developed for the quantification of resiniferatoxin (RTX) in canine cerebrospinal fluid (CSF). A reversed-phase C_{18} column and acetonitrile in 0.02 *M* NaH_2PO_4 as mobile phase provided satisfactory resolution for RTX analysis. Direct HPLC analysis of the CSF samples without sample extraction or preparation improves the accuracy and detection limits of this assay. This assay was applied to measure CSF RTX content to test this method for research and clinical applications related to studies examining its analgesia effects.
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1. Introduction

Resiniferatoxin (RTX), a highly potent capsaicin analog, is an activator of the vanilloid receptor-1 (VR1) [1]. Neurons expressing VR1 are thought to be involved in nociception throughout the body including the afferent pathways from the bladder. Clinical trials using intravesicle RTX has provided extended relief of symptoms related to overactive or hyperreflexic bladder [2,3]. VR1 agonists are also currently used as topical agents for neuropathic pain. Repeated application of capsaicin diminishes pain symptoms reported by these patients [4]. While both capsaicin and RTX have shown clinical promise in animal pain models, RTX was more potent and has reduced side-effects and could therefore be a better therapeutic option [5].

An accurate and sensitive method for measuring RTX concentrations in biological fluid was necessary for performing pharmacokinetic and pharmacodynamic studies to assess the therapeutic efficacy and physiological actions of the drug in clinical trials. No assay technique for quantifying RTX in biologic fluids has been reported to date. CSF was evaluated

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because it is a possible route of administration for RTX in patients with advanced metastatic disease. In this study, a simple and effective HPLC method to measure RTX concentrations in canine CSF samples was developed using a reverse phase C_{18} column. The procedure allowed direct injection of samples into the HPLC without sample preparation thus permitting the rapid analysis of large numbers of samples. Specific modifications to enhance the separation of RTX from interfering compounds were developed to improve the detection limits of the assay. Thus, this novel method allows for accurate quantification of low therapeutic concentrations of the RTX in clinical specimens.

2. Experimental

2.1. Reagents and materials

Resiniferatoxin ($C_{37}H_{40}O_9$, M_W=628.73) for the standards (RTX-s) was supplied by Sigma (St. Louis, MO, USA) (see Fig. 1). Clinical grade resiniferatoxin (RTX-c) was purchased from LC Laboratories (Woburn, MA, USA) and prepared and packaged by the Pharmaceutical Development Section, NIH (Bethesda, MD, USA). All solvents used were of HPLC grade and all other chemicals were of analytical grade (Sigma). The HPLC system consisted of an LC-600 pump, a variable wavelength UV detector SPD-6AV set at 252 nm (Shimadzu; Kyoto, Japan), a SIL-10A autoinjector, a one-channel recorder Kipp and Zonen BD 40, a column heater (Alltech Associates; Deerfield, IL, USA), and an analytical C_{18} column (250×4.6 mm I.D., 5 μm particle size) (Supelco; Bellefonte, PA), USA.

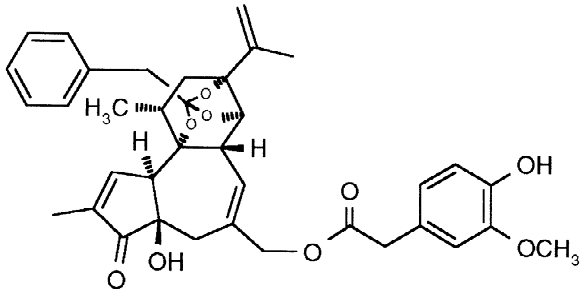

Fig. 1. Structure of resiniferatoxin ($C_{37}H_{40}O_9$, M_W=628.73).

2.2. Sample preparation

The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania prior to the start of the study. Dogs weighing between 8 and 20 kg were anesthetized and intrathecal catheters were placed at both the lumbar and cervical levels. The animals were recovered and observed for changes in gross behavior. The following day, the animals were anesthetized and patency of both catheters tested by withdrawing CSF. A single injection of RTX-c (1.0 or 10 μg/kg) was administered via the lumbar catheter and then 0.25 ml CSF samples were drawn from the cervical catheter prior to and 5, 10, 20, 30, 60, 120 and 240 min after the injection. Samples were frozen and stored at −80 °C until processing. CSF was spun at 10 000 *g* for 5 min and the supernatant transferred to a clean tube on ice.

2.3. Standard solutions

A fresh solution of RTX was prepared in methanol at a concentration of 1 mg/ml and six standards solution for calibration containing 0.5–8000 ng/ml of RTX-s were prepared by serial dilutions in the HPLC mobile phase. Calibration curves were constructed by plotting the height of the RTX peak against the known concentration of RTX in the standard and fitted by linear regression analysis.

Quality control samples with RTX concentrations of 10 and 400 ng/ml were prepared by adding RTX standard solution to human CSF and stored at −80 °C. Because there was no difference seen when using either CSF (either dog or human) or HPLC mobile phase as solvent to prepare RTX samples (data not shown), HPLC mobile phase was used to prepare the standard solution and human CSF was used to prepare quality control samples for experimental convenience. For each validation run, duplicate quality control samples were thawed and assayed.

2.4. HPLC conditions

HPLC was carried out at a flow-rate of 1 ml/min and at a temperature of 45 °C with an isocratic

mobile phase of acetonitrile–0.02 *M* NaH_2PO_4 (32:50, v/v; pH 4.3).

2.5. Accuracy and precision

The chromatographic peak for RTX was identified by retention time and standard addition protocols [6,7]. RTX was assayed by measuring the chromatographic peak heights and drug concentrations determined from the standard calibration curve that was prepared daily. Quality control samples with RTX concentrations of 10 and 400 ng/ml were used to determine the inter- and intra-assay variability. Variability was expressed as standard error of the mean (±SEM). Relative standard deviation (RSD) and relative error (RE) were determined for inter- and intra-assay variation.

3. Results

Clear separation and a distinct chromatographic peak at 6.5 min were achieved under our HPLC conditions using samples collected after the RTX-c injection (Fig. 2a). Fig. 2b revealed a clean ''window'' at 4.9–7.5 min in the reagent blank for CSF RTX sample. The additional clinical drug was injected into HPLC and the same chromatographic profile was found. The canine CSF sample used above spiked with 20 ng of the internal standard RTX-s resulted in an increased height of the peak seen in the clinical samples (Fig. 2c). A control CSF sample spiked with the drug standard (RTX-s) resulted in a single peak height at 6.5 min, further confirming the identification of analyte (data not shown).

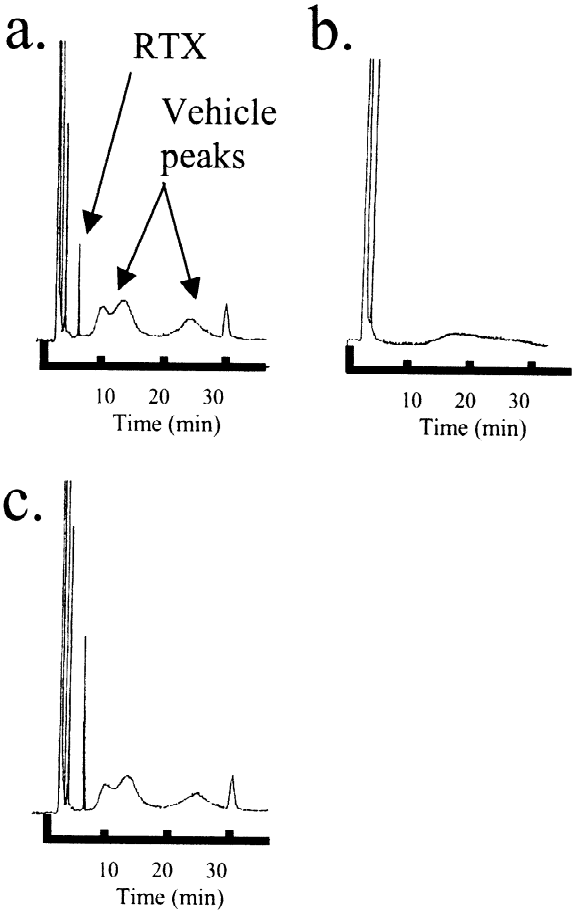

Fig. 2. (a) A chromatogram of canine CSF sample obtained from 10 μg/kg administration of RTX-c. (b) A chromatogram of the CSF sampled prior to the administration of RTX (same animal). The RTX peak and vehicle peaks are not seen. (c) The sample used to generate the chromatogram in panel a was spiked with 20 ng of standard RTX-s. The resulting chromatogram shows only an increase in the height of the single peak specific to the analyte RTX.

3.1. Variability

The mean (±S.E.M.) measured RTX concentration of six CSF samples containing 10 and 400 ng/ml of RTX injected into the HPLC for analysis was 9.81±0.25 and 393±9.1 ng/ml, respectively. The same samples were assayed on 6 different days using the same method. The concentration remained constant and was 9.77±0.31 and 391±12.00 ng/ml, respectively, when assayed at the later time periods. RSD and RE for the intra- and inter-day assays are presented by sample with high and low concentrations as shown in Table 1. Quality control samples with RTX were stored at −80 °C for 4 months and were assayed as described and similar results were obtained. These data demonstrate the long-term stability of RTX in CSF under the experimental conditions described despite RTX's air and light sensitivity.

Table 1
Intra- and inter-day RTX assay in canine CSF

	Intra-day ($n=6$)		Inter-day ($n=6$)	
Mean±SD (ng/ml)	9.81±0.25	393±9.1	9.77±0.31	391±12.0
RSD[a] (%)	2.6	2.3	3.2	3.1
RE[b] (%)	1.9	1.8	2.3	2.3

Ten and 400 ng/ml of RTX-s were added to canine CSF and injected in the HPLC for analysis. The mean, standard deviation (SD), intra- and inter-day RE and RSD were calculated.

[a] Relative standard deviation (RSD) = SD/mean × 100.

[b] Relative error (RE) = (mean − nominal)/nominal × 100.

3.2. Standard curve and sensitivity

The chromatographic peak height for RTX was linear with CSF drug concentrations in the range of 0.5–8000 ng/ml. The linear regression equation for RTX standard curves was $y = 1.328x + 0.8$ ($R^2 = 0.9999$). The standard error of slope and intercept of each standard curve was less than 2% of the response obtained for a targeted RTX concentration in the range of the calibration curve. A quantitation limit of detection of 0.1 ng/ml for RTX was determined by assuming a concentration of the analyte that yielded a signal-to-noise ratio of 5. Fig. 3 shows a typical pattern of RTX concentrations in canine CSF solution ($n=1$/dose) after the respective intrathecal injection (1.0 or 10 μg/kg) of RTX at different time points. CSF levels of RTX constantly decreased, indicating the redistribution or metabolism–catabolism of RTX after administration.

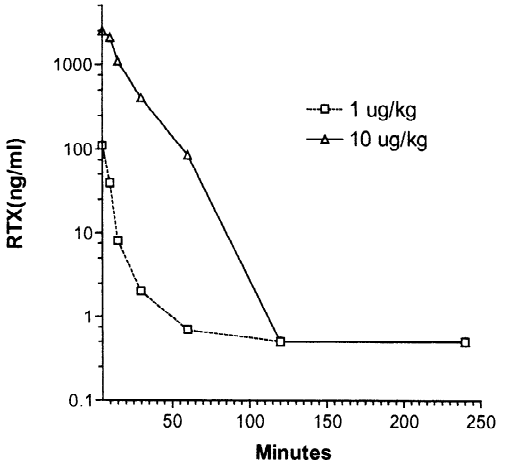

Fig. 3. Canine CSF concentrations at different time points. CSF was withdrawn from the catheter prior to and 5, 10, 20, 30, 60, 120 and 240 min after the injection of either 1 or 10 μg/kg RTX. One dog was used at each dose and CSF RTX was measure in triplicate using the HPLC assay.

4. Discussion

Common methods of sample preparation for HPLC analysis are the liquid–liquid (LPE) and solid-phase extraction (SPE) techniques in which interfering materials are removed from the sample to decrease baseline noise and extraneous peaks and thus enhance signal detection. There is an inherent discrepancy or variation of the internal standard and analyte due to the loss from sample preparation–extraction. In addition, RTX is both air and light sensitive making any extraction procedure more difficult. As previously described [8], CSF samples can be directly analyzed by HPLC because CSF contains only low concentrations of peptides, proteins and other interfering substances. In the present study, injecting the CSF sample into a HPLC column for direct analysis eliminated the need for sample extraction and improved the sensitivity and reliability of data. Several hundred samples were analyzed using the HPLC assay in our lab with no apparent degradation of chromatographic performance.

Several columns and concentrations of acetonitrile were explored while optimizing this assay. A C_{18} column and acetonitrile in the mobile phase were chosen for measuring the samples because of the hydrophobicity of RTX. Increasing the concentration of acetonitrile better separated the RTX peak from two additional late peaks observed during elution of

the sample. These peaks were likely derived from other substances used to prepare the clinical drug (including Tween-80) since they were seen when comparing the HPLC profile between canine CSF samples and CSF samples spiked with the clinical grade drug. Due to the clean separation, the late-eluting peaks with higher retention times than RTX were not taken into account and did not affect HPLC analysis. CSF samples spiked with RTX-s resulted in increased amplitude of the parent compound (RTX) specific single peak height at 6.5 min with no increase of the vehicle peaks.

This assay is being used in our laboratory to quantify the concentration of RTX in canine CSF after a single injection of RTX. While the animal numbers included are limited, this assay is currently being used to characterize the pharmacokinetic properties of the drug in a large animal model. Satisfactory separation with great reproducibility showed that the RTX concentration in CSF could be measured with a high degree of confidence.

Acknowledgements

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