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Measurement of resiniferatoxin in serum samples by high-performance liquid chromatography

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Abstract

A novel and simple method of extraction, separation, identification and quantification of resiniferatoxin (RTX) in serum samples is reported. Human serum and whole blood were treated with acetonitrile to denature proteins, such as orosomucoid, and the soluble fraction was passed through a reversed-phase C_{18} cartridge. RTX eluted from the cartridge was quantified by high-performance liquid chromatography (HPLC) using a reversed-phase C_{18} column. Reproducible recovery of RTX and tinyatoxin, an internal standard, from serum was achieved. Isocratic elution with 62% acetonitrile provided a suitable retention time without interfering peaks eluting near the analyte. Therefore, the procedure described provides a useful assay for determination of serum RTX pharmacokinetic parameters.
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1. Introduction

Resiniferatoxin (RTX) is a highly potent capsaicin analog and an activator of the vanilloid receptor-1 (VR-1, TRPV1), a ligand-gated ion channel highly expressed in nociceptive primary sensory neurons. Clinical trials using intravesical injections of RTX have been conducted to evaluate its use in several diseases that result in debilitating bladder symptoms. These studies have shown that RTX is effective in providing prolonged relief from spasm and pain [1]. While both capsaicin and RTX were shown to be effective in other pain states, RTX is more potent, has reduced side effects, and may therefore be a better therapeutic option. We have previously described a high-performance liquid chromatography (HPLC)-based assay to measure RTX in canine cerebrospinal fluid [2]. Unlike serum, the cerebrospinal fluid is relatively protein-free and RTX was assayed without the need for an extraction step.

To our knowledge, an accurate and sensitive method for measuring RTX concentrations in other biologic fluids, including blood, has not been described. Similar to other drugs, vanilloids (including RTX) have a strong affinity for orosomucoid (alpha-1-acid glycoprotein, AGP), complicating its quantification in serum [3,4,7]. To obtain preclinical data in animals and humans, it is necessary to develop assay techniques suitable for serum. In this study, an HPLC method to measure RTX concentration in serum was developed which is suitable for rapid, high-throughput analysis. RTX was effectively separated from other UV-absorbing material leading to a simple, highly accurate assay. This novel method for sample preparation, identification and quantification of RTX was further applied to characterize RTX distribution in human whole blood.

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2. Experimental

2.1. Reagents and materials

Human serum, RTX (purity >96%) and tinyatoxin (as an internal standard, purity >98%) were obtained from Sigma (St. Louis, MO, USA; see Fig. 1 for structure and MW). Chlorpromazine and tris-butoxyethylphosphate were purchased from Aldrich (Milwaukee, WI, USA). C_{18} solid phase extraction (SPE) cartridges and SPE column processors were obtained from J.T. Baker (Phillipsburg, NJ, USA). All solvents used were of HPLC grade and all other chemicals were of analytical grade (Sigma).

2.2. Apparatus

The HPLC system consists of a LC-600 pump, a variable wavelength UV detector (SPD-6AV, Shimadzu, Kyoto, Japan) set at 252 nm, a SIL-10A autoinjector (Shimadzu), a one-channel recorder (Kipp and Zonen BD 40, Rotterdam, the Netherlands), and a reversed-phase column (Supelcosil LC-PAH #58229; 250 mm × 4.6 mm i.d., 5 μm particle size, Supelco, Bellefonte, PA, USA).

2.3. Solid-phase extraction (SPE)

An internal standard consisting of 120 ng tinyatoxin in 10 μl methanol was added to 1 ml of the appropriate serum sample. Following the addition of 1 ml acetonitrile, the samples were vortexed for 30 s and centrifuged at 15,000 × *g* for 30 min at 4 °C. Soluble fractions were passed through a C_{18} SPE cartridge that was pre-conditioned with 2 ml methanol and equilibrated with 2 ml 20 mM phosphate buffer (pH 7.5). The cartridge was washed with 2 ml 20 mM phosphate buffer and the samples were then eluted with 0.5 ml methanol containing 0.5% triethylamine (TEA). A 150 μl aliquot of each sample was injected into the HPLC system for analysis.

2.4. Standard solutions

A stock solution of RTX was prepared in methanol at a concentration of 1 mg/ml and stored at −80 °C. On each day the assay was performed, six standard solutions for calibration (5, 10, 20, 50, 400 and 800 ng/ml of RTX) were prepared by serial dilution of the stock solution in methanol. The serial dilution selected spans the expected experimental clinical range. Calibration curves were constructed by plotting the height of the RTX peak against the known concentration of RTX in the standard and then fitted by linear regression analysis.

Quality control samples with RTX concentrations of 10 and 40 ng/ml were prepared by adding a RTX standard solution to human serum and storing at −80 °C. For each validation run, duplicate quality control samples were thawed and assayed.

2.5. Recovery

The recovery of RTX and internal standard from SPE was estimated by extracting standard serum samples containing known concentrations of RTX (50, 100 and 500 ng/ml) and tinyatoxin. Blank samples were prepared by adding the equivalent volume of solvent without drug to 1.0 ml serum. Samples were analyzed to verify that no peaks appeared near the retention times of either RTX or tinyatoxin in the final HPLC experiments. The percent recovery of RTX and tinyatoxin was determined by comparing the height of the analyte peak measured in the extracted standards to the height of the analyte peak measured directly in the spiked samples containing the known amount of analyte.

2.6. HPLC conditions

A conventional C_{18} reversed-phase column protected by a C_{18} guard cartridge (Supelco-see above) was used for the study. The column was pre-equilibrated with 62% (v/v) acetonitrile in 20 mM NaH_2PO_4 buffer (pH 4.6) and a 150 μl sample volume was injected. All HPLC separations were carried out at a flow rate of 1 ml/min (45 °C) with isocratic elution using 62% (v/v) acetonitrile in 20 mM NaH_2PO_4 buffer (pH 4.6) as the mobile phase.

2.7. Accuracy and precision

The chromatographic peaks for RTX and tinyatoxin were identified by retention time in comparison to standard solutions of RTX and tinyatoxin [5,6]. RTX concentration was determined by measuring the chromatographic peak height and comparing that value to a standard calibration curve that was prepared daily. For every validation run, the recovery of RTX from each serum sample was determined using tinyatoxin as the internal standard. Quality control samples with

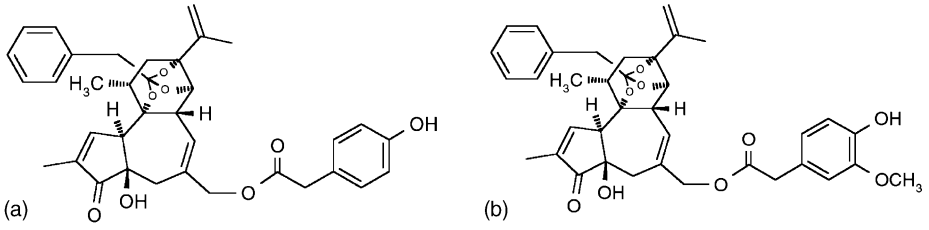

Fig. 1. Structure of tinyatoxin and resiniferatoxin (RTX). (a) tinyatoxin ($C_{36}H_{38}O_8$, MW = 598.7); (b) resiniferatoxin ($C_{37}H_{40}O_9$, MW = 628.7).

Table 1
Recovery of RTX from C_{18} cartridge extraction

Serum RTX (ng/ml)	Extracted RTX (ng/ml ± S.E.M., $n = 6$)	%Recovery
50	48.9 ± 0.7	97.8
100	92.0 ± 1.5	92.0
500	479.8 ± 2.0	96.0

an RTX concentration of 20 or 100 ng/ml ($n = 6$ per concentration) were used to determine the inter- and intra-assay variability. Data are reported as mean ± standard error of the mean (S.E.M.). Coefficients of variation were determined for inter- and intra-assay variation.

2.8. Human whole blood analysis

To determine the distribution of RTX in blood, the components were examined separately. Human whole blood (5 ml) was collected in a heparinized tube and 20 μg RTX was added. After vortexing, the plasma was separated from the cellular components by centrifugation at 3000 × *g* for 20 min at 4 °C, and serum RTX was analyzed as described above. To assay RTX in the red blood cell compartment, packed red blood cells from the above centrifugation (0.5 ml) were added to 0.5 ml phosphate buffer (pH 7.5) and homogenized using a Polytron homogenizer. One ml of acetonitrile was added to each sample and then centrifuged (15,000 × *g* for 30 min at 4 °C). The supernatant was subjected to SPE and HPLC analysis as described above.

3. Results

3.1. Solid-phase extraction

The C_{18} cartridge extraction method, once optimized, resulted in efficient recovery of the RTX from the human serum samples. Serum samples containing 50, 100 or 500 ng RTX/ml averaged greater than 95% drug recovery (see Table 1). Recovery of tinyatoxin was similar to RTX (data not shown). Eluted serum samples subsequently spiked with standards (10 or 40 ug/ml) prior to injection into the HPLC system resulted in increased amplitude of the respective RTX or tinyatoxin single peak height, confirming the identification of each analyte. A high pH elution buffer caused lower recovery of RTX from the SPE procedure and a washing buffer of pH 7.5 for sample preparation achieved the optimal recovery of RTX (data not shown).

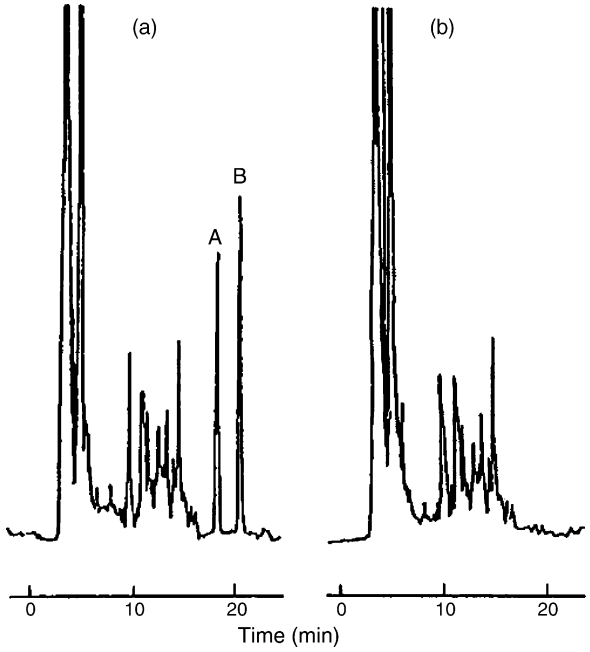

Fig. 2. Typical chromatogram of serum sample. (a) Serum 50 ng/ml RTX; 100 μl (representing 5 ng RTX) was injected into the HPLC for analysis. A: RTX, B: internal standard tinyatoxin (12 ng); (b) Serum blank: 1 ml extracted by SPE and 150 μl injected into the HPLC for analysis.

3.2. Accuracy and precision

3.2.1. Standard curve

The chromatographic peak height for RTX was linear relative to a serum drug concentration range of 5–800 ng/ml. The RTX standard curve was fit by linear regression with the equation $y = 1.435x + 0.8$ ($r^2 = 0.9999$).

3.2.2. Intra- and inter-day RTX assay

Intra-day and inter-day RTX serum samples with concentration of 20 or 100 ng/ml ($n = 6$ per concentration) were assayed using the described methods. The RTX mean concentrations (±S.E.M.) and coefficient of variation are shown in Table 2.

3.2.3. Selectivity

A distinct chromatographic peak for both RTX and tinyatoxin and a clear separation from other UV absorbing serum constituents was achieved under our HPLC conditions (Fig. 2a). Fig. 2b reveals that no UV absorbing peaks with the retention time of RTX and tinyatoxin were observed in the serum blank. The same extracted serum sample from

Table 2
Intra- and Inter-day RTX assay in human serum

	Intra-day ($n = 6$)		Inter-day ($n = 6$)	
Mean ± S.E.M (ng/ml)	18.9 ± 0.2	99.3 ± 0.4	18.1 ± 0.3	97.1 ± 0.7
Coefficient of variation (%)	1.06	1.13	1.66	0.72

Twenty and 100 ng/ml RTX were added to human serum, subjected to SPE and injected in the HPLC column for analysis. The mean, standard error of the mean (S.E.M.), intra- and inter-day coefficient of variation were calculated.

Fig. 2a spiked with 20 ng RTX standard solution resulted in an increased height of only the single peak specific to the analyte (not shown).

3.3. Limit of detection

The limit of detection for this method is 0.4 ng/ml for RTX (signal-to-noise ratio = 5). Typically only 25% of each sample volume was injected into the HPLC column for measurement. The level of sample detection potentially could be greater if more of the eluted sample is assayed. Preliminary experiments to concentrate the sample volume by evaporating the methanol under a stream of nitrogen gas and using the reduced volume for HPLC analysis resulted in a proportionally larger peak (not shown). Concentrating the other components of the serum still resulted in clear separation from other UV peaks and had no effect on sample analysis.

3.4. Whole blood processing

The 5 cc human whole blood samples spiked with 20 μg RTX yielded 3 cc of plasma, corresponding to a normal hematocrit of 40%. The amount of RTX measured from the 3 ml plasma after HPLC analysis was 19.6 μg. Three additional human blood samples were tested with similar results (not shown). In contrast, no RTX was found in blood cell homogenates indicating that RTX, even in excess (well above the concentrations added previously to serum), does not bind appreciably to the red blood cells. Fig. 3a shows the chromatogram of the plasma sample with a similar clear separation from other UV absorbing constituents seen with human serum. Thus, either plasma or serum could be utilized with this SPE and HPLC method.

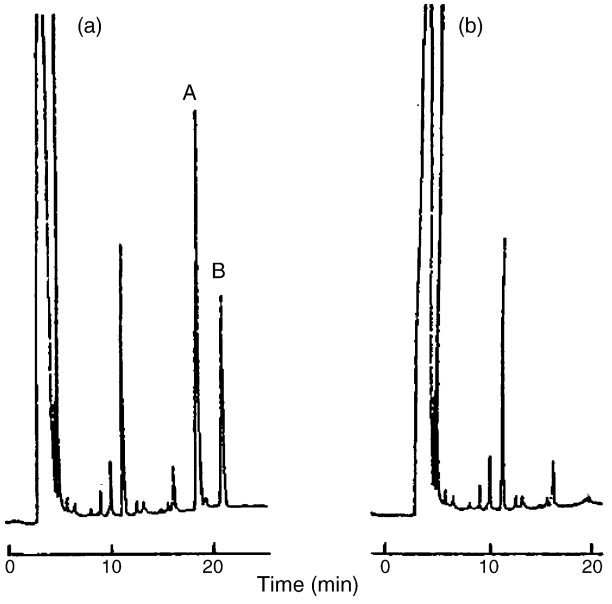

Fig. 3. Typical chromatogram of a human plasma sample. (a) Plasma from 5 ml whole blood spiked with 20 μg RTX; 20 μl of elute from the SPE cartridge was injected into the HPLC for analysis. (A: RTX, B: internal standard tinyatoxin); (b) plasma blank: 1 ml plasma extracted by SPE and 150 μl injected into the HPLC for analysis.

4. Discussion

The data presented described an efficient method for the extraction and quantification of RTX in human blood. To evaluate the SPE procedure for RTX recovery from blood, a reversed-phase C_{18} cartridge was used to pre-fractionate the serum prior to HPLC. Direct extraction of serum with the C_{18} cartridge was problematic due to high affinity RTX binding to the plasma protein AGP, resulting in low recovery [7]. RTX–AGP could have been washed out or not eluted from the C_{18} cartridge during the extraction procedure, also resulting in reduced recovery. As an initial alternative approach, the most potent inhibitors of RTX–AGP binding (chlorpromazine and trisbutoxyethlphosphate) were added to the serum samples prior to extraction to compete for binding sites. There was no significant increase in RTX recovery, suggesting that AGP is not the only binding site. RTX may bind to other endogenous substances, such as bilirubin, urea, creatinine, [8] and white blood cells (monocytes, granulocytes, and monocytes) [9].

To minimize RTX binding affinity to orosomucoids in plasma, the extraction procedure was modified by acidification with 20 μl H_3PO_4 added to 1 ml serum. As a result, the recovery increased significantly (>90%). This suggests that RTX couples with other compounds in serum and co-elutes during the washing procedure, consistent with the literature [7]. However, the subsequent HPLC analysis showed a very high background from co-eluted materials after the methanol elution step, which strongly affected the quantification of RTX. Therefore, acetonitrile was subsequently chosen to denature orosomucoid proteins before the extraction procedure. The denaturing step greatly increased the amount of free RTX and eliminated most proteins, resulting in high drug recovery and low interfering background in HPLC analysis.

Tinyatoxin was selected as the internal standard for its structural similarity (see Fig. 1) and compatible recovery with respect to RTX. The single methoxy group difference combined with comparative chromatographic behavior demonstrated their similar physio-chemical properties, making the choice of tinyatoxin as the internal standard reasonable.

A high percentage of acetonitrile in the HPLC mobile phase was optimal for measuring RTX since co-extracted substances present in serum were washed out first. RTX's hydrophobicity and strong interaction with the stationary phase of the C_{18} column provided an optimal capacity factor, producing clean resolution of the analyte peaks. This was demonstrated by spiking the serum samples with an RTX standard before HPLC separation. Additionally, separating plasma from human whole blood was evaluated using the extraction procedure and HPLC analysis with similar results.

The success of this method results largely from denaturing plasma-binding proteins with acetonitrile, providing high extraction recovery by increasing the concentration of free RTX. By eliminating plasma proteins, the background after subsequent HPLC analysis is greatly reduced. RTX has been proposed to be used for controlling severe pain by a cell deletion mechanism. However, more work is needed to better understand the metabolism and distribution of the drug to body compartments. These methods will facilitate the clinical implementation of RTX in human or veterinary applications [10].

References

[1] M.B. Chancellor, Urology 55 (5A Suppl.) (2000) 65.
[2] A.J. Mannes, D.C. Brown, S.Z. Perkowski, J. Keller, R.M. Caudle, M.J. Iadarola, Q.C. Meng, J. Chromatogr. B 780 (3) (2002) 475.
[3] Z.H. Israili, P.G. Dayton, Drug Metab. Rev. 33 (2) (2001) 161.
[4] T. Fournier, N. Medjoubi-N, D. Porquet, Biochim. Biophys. Acta 1482 (2000) 157.
[5] P. Weiss, R.M. Hersey, C.A. Dujovne, J.R. Bianchine, Clin. Pharmacol. Ther. 10 (1969) 401.
[6] G.A. Gerhardt, C.J. Drebing, R. Freedman, Anal. Chem. 58 (1986) 2879.
[7] A. Szallasi, N.E. Lewin, P.M. Blumberg, J. Pharmacol. Exp. Ther. 262 (1992) 883.
[8] J.P. Tillement, G. Houin, R. Zini, S. Urien, E. Albengres, J. Barre, M. Lecomte, P. D'Ahis, B. Sebille, Adv. Drug Res. 13 (1984) 59.
[9] G.C. Gahmberg, L.C. Andersson, J. Exp. Med. 148 (2) (1978) 507.
[10] L. Karai, D.C. Brown, A.J. Mannes, S.T. Connelly, J. Brown, M. Gandal, O.M. Wellisch, J.K. Neubert, Z. Olah, M.J. Iadarola, J. Clin. Invest. 113 (2004) 1344.