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Determination of sodium 3,4-diaminonaphthalene-1-sulfonate, a Congo Red derivative, in plasma and brain of hamsters by high-performance liquid chromatography after solid-phase extraction and ultraviolet absorbance

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Abstract

In the search for compounds with similar or greater activity than Congo Red (CR) in protecting normal prion protein from being converted into the pathological form, we have synthesized various compounds which derive from CR. One of these is the sodium 3,4-diaminonaphthalene-1-sulfonate (RCA) which has an activity similar to CR in preliminary experiments. This study describes a method to determine RCA in plasma and in brain tissue by high-performance liquid chromatography (HPLC), using a solid-phase extraction and UV detection. RCA is an amphoteric molecule difficult to separate from biological matrices. Extraction was achieved by solid-phase extraction (ENV+ columns) together with the use of a counter ion. The resulting solid-phase extraction is efficient and rapid. RCA was separated on a Symmetry C_{18} 250×4.6 mm I.D. 5 μm column at 1 ml/min using a 50 m*M* Na_2SO_4 in 5 m*M* tetra-*n*-butylammoniumiodide (TEBA) in water–methanol (82:18, v/v) mobile phase. Retention times of RCA and I.S. were 21 and 24 min. The UV detector was set at 210 nm. The limit of quantitation was 0.5 μg/ml. The method has intra-assay and inter-assay accuracies higher than 95%, coefficients of variation ranging between 2.8 and 8.6%, and recovery rates between 74.3 and 80.1% in plasma and in brain tissue. A linear response to quantities of RCA from 0.5 to 100 μg/ml or 10 μg/g in plasma or brain was obtained. The present method allows the study of the pharmacokinetics of RCA in plasma after i.p. administration, and the distribution of the compound into the brain at the peak time.

Keywords: Sodium 3,4-diaminonaphthalene-1-sulfonate

1. Introduction

Congo Red (CR) is a sulfonated azo dye widely used for biological staining which has recently been the focus of considerable interest. In fact, it has been shown to bind to proteins involved in viral recognition and replication [1]. CR has long been employed in histological stains of amyloid proteins of the type

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found in neurodegenerative conditions such as Alzheimer's disease, transmissible spongiform encephalopathies (TSE) in many species and scrapie in sheep [1]. It has also been demonstrated to protect normal prion protein from being converted to the protease-resistant form, an important step in the pathology of the so-called viral diseases [2–4]. CR binds to a wide range of biological molecules and it is therefore an important lead compound in drug development such as new anti-HIV, anti-Alzheimer's and anti-TSE therapeutic agents.

We were particularly interested in the ability of CR to potently inhibit the accumulation of the scrapie-associated, protease resistant isoform of PrP protein without affecting the normal isoform [5]. Preliminary experiments in hamsters infected with a strain specific PrP(Sc) capable of inducing a spongiform encephalopathy, confirmed the ability of CR to protect from disease progression [6,7]. In the search for compounds with similar or greater activity than CR but with lower toxicity, we have synthesized various compounds which derive from CR but do not possess the central benzidinic part of its molecule. One of these is the reduced derivative of CR, the sodium 3,4-diaminonaphtalene-1-sulfonate (RCA) which exhibited activity similar to CR in experiments ''in vitro'' and in the hamster TSE model [7–10]. The study of the pharmacokinetics of this compound and its tissue distribution may be relevant to the understanding of the targets of its action and its mechanism of inhibiting prion infection. RCA has amphoteric properties due to the presence of basic (amino) and strong acidic (sulfonate) groups: whereas there are some studies in the literature which describe the analytical method for the determination of compounds with such properties, there are no studies which describe the separation of aromatic amino-sulfonated compounds from biological matrices [11–13].

This study describes a method to determine RCA in plasma and in brain tissue by HPLC, using a solid-phase extraction and UV detection.

2. Experimental

2.1. Chemicals and reagents

RCA (sodium 3,4-diaminonaphthalene-1-sulfonate; Fig. 1) and sodium 4-aminonaphthalene-1-sulfonate (internal standard, I.S.; CAS: 130-13-2), were synthesized from the Pharmaceutical and Toxicological Chemistry Institute of the Faculty of Pharmacy. Methanol was HPLC-grade, tetra-*n*-butylammoniumiodide (TEBA), sodium disulfite ($Na_2S_2O_5$), sodium sulfate (Na_2SO_4), zinc sulfate ($ZnSO_4 \times 7H_2O$), barium hydroxide ($Ba(OH)_2 \times 8H_2O$) and cysteine hydrochloride were of analytical grade (Merck, Darmstadt, Germany).

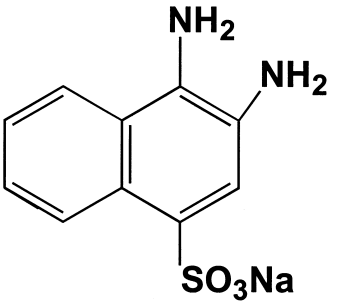

Fig. 1. RCA structure formula.

Isolute™ liquid–solid extraction columns, packed with 50 or 200 mg of ENV+ hydrophobic phase and with 1 or 3 ml reservoir (International Sorbent Technology LTD, Mid Glamorgan, UK) were used to process the plasma or brain tissue samples, respectively.

Stock solutions of drugs (1 mg/ml) in 1% sodium disulfite (antioxidant) in water were prepared weekly and stored at 4 °C protected from light. Standard working solutions were prepared daily immediately before use in 1% sodium disulfite in water. Phosphate buffer was 50 m*M* KH_2PO_4/Na_2HPO_4, pH 5.

2.2. Determination of the RCA stability in solution

RCA is a diamino sulfonated compound and may be very unstable at room temperature when in solution, possibly because the oxidization of the amino groups.

To determine the stability of the compound in solution, three solutions of RCA at 10 μg/ml, one in phoshate buffer pH 5.0, one in buffer and bubbling N_2 (at a flow of 1 ml/min) and one in buffer and

sodium disulfite 1% were kept at room temperature and 100 μl were injected immediately and at 30 min intervals thereafter up to 180 min.

2.3. Biological samples

Plasma and brain tissue were obtained from syrian hamsters treated intraperitoneally (i.p.) with 100 mg/kg of RCA. Before and 15, 30, 45, 60, 90 min after administration, the animals were anesthetized with diethyl ether and blood was drawn by heart puncture, centrifuged and plasma stored at −25 °C until analysis. After killing the animal the brain was removed, washed with physiological solution with 1% sodium disulfite, wiped with clean paper, weighed and frozen at −25 °C.

At the beginning of the study, three series of standard plasma and tissue samples of non treated animals, prepared as described below, spiked with low, medium and high concentrations of RCA (1, 10, 100 μg/ml in plasma, 1, 2, 5 μg/g in brain tissue; quality control samples) were frozen at −25 °C and then one of each concentration analyzed with each run of the unknown samples. Samples were analyzed within 2 weeks.

2.4. Extraction of RCA from plasma and brain tissue

Ten micrograms of I.S. and 1 ml of 1% sodium disulfite in 5 m*M* TEBA in water were added to 1 ml of plasma. After vortexing for 1 min and centrifuging for 10 min at 800 *g* the sample was applied to an Isolute ENV+ column 50 mg placed on a Vac-Elut apparatus connected to a vacuum.

A hamster brain (about 0.9 g of tissue) was placed in a 20 ml plastic tube containing 4 ml of 6% cysteine in 0.4 *M* $ZnSO_4$ in water and 10 μg of I.S.: after homogenization in the Ultra-Turrax apparatus (IKA-Werk GMBH and CO KG, D-79219, Staufen, Germany), 4 ml of 0.4 *M* $Ba(OH)_2$ and 2 ml of 5% sodium disulfite in 5 m*M* TEBA in water were added. Samples were centrifuged for 10 min at 30 000 *g* and 10 ml of the clear supernatant applied to a 200 mg Isolute ENV+ column placed on a Vac-Elut apparatus connected to vacuum.

Before applying samples, columns were washed, with the vacuum on, with one column volume of methanol, followed by one volume of water and one column volume of 1% sodium disulfite in 5 m*M* TEBA in water. The vacuum was then turned off and, without allowing the column to dry, the sample was added to the column and the vacuum turned on. The columns were then washed with two column volumes of 1% sodium disulfite in 5 m*M* TEBA in water and one column volume of water–metanol (90:10, v/v). The columns were left to dry for 5 min and then the vacuum was turned off. A 12 ml conical centrifuge tube was placed in the VacElut rack under each column. With the vacuum off, 500 μl of methanol were added to each column and the vacuum turned on until all the eluate was collected, and then turned off; a second aliquot of 500 μl of methanol were added to each column and the vacuum turned on until all the eluate was collected with the first. The combined eluates were evaporated to dryness at 25 °C under a low nitrogen flow, the residue was reconstituted in 0.5 ml of mobile phase and 50–100 μl injected into the chromatograph.

Standard samples were prepared by spiking 1 ml of drug-free plasma or the drug-free brain with known amounts of RCA (0.5–100 μg in plasma; 0.5–10 μg in brain) and 10 μg of I.S.. The samples were analyzed according to the procedure described above.

Calibration curves were calculated by linear regression analysis of the ratios of the area of the peak of RCA and those of I.S. versus the RCA concentrations in the standard samples. These curves were used to calculate the concentrations of RCA in unknown samples.

2.5. Liquid chromatography

HPLC analysis was carried out using a Shimadzu (Kyoto, Japan) system: LC-6A HPLC pump, SPD-10A UV detector, SIL-6A Autosampler and SCL-6A CR4-A Controller Integrator. Wavelength was set at 210 nm. Separation of RCA from the I.S. and the interfering peaks of plasma and brain tissue was achieved on a Symmetry® C_{18}, 250×4.6 mm I.D., 5 μm column (Waters, Milford, MA, USA), with a 2-cm pre-column filled with the same material. Elution of the compounds was performed at 1 ml/

min using 50 m*M* Na_2SO_4 in 5 m*M* TEBA in water–methanol (82:18, v/v) as mobile phase.

2.6. Accuracy and precision

Accuracy and precision were evaluated with values obtained following analyses of five standard samples replicated ($n=10$) on the same day (intra-day accuracy and precision) and following daily analyses of three quality control standard samples at three different concentrations (low, medium and high; 1, 10, 100 μg/ml in plasma, 1, 2, 5 μg/g in brain tissue; inter-day accuracy and precision). Accuracy was calculated as percentage of the measured vs. the known concentrations. Precision was determined as the coefficient of variation, (C.V.), i.e. the ratio between the mean of the found concentrations and its standard deviation (SD).

2.7. Recovery

The absolute recovery of RCA from plasma and tissue samples was determined by comparing the areas of the peaks of standards not extracted (prepared in the mobile phase) with those of standards at the same concentrations extracted according to the procedures described above.

3. Results

3.1. Stabilty of RCA in solution

RCA decays quite rapidly as shown in Fig. 2: the decay is partially prevented by removing oxygen from the solution with nitrogen and completely prevented by adding an antioxidant, 1% sodium metabisulfite.

Therefore in order to prevent the decay during sample preparation, 1% sodium disulfite had to be added both to the samples and to the solutions employed for the extraction procedure. In these conditions extracts and biological sample have found to be stable over 24 h at room temperature.

No decay was found on the analytical column since no change of the area under the peak of the RCA was observed after adding the antioxidant (sodium disulfite 1%) to the mobile phase.

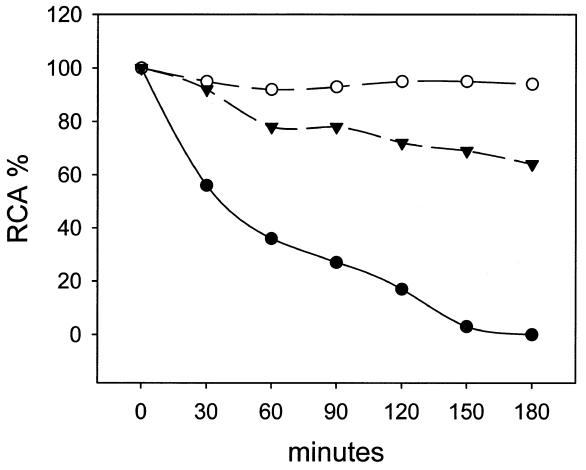

Fig. 2. Stability of RCA in solution (10 μg/ml) at 37 °C: filled circles, pH 5.0 phosphate buffer; filled triangles pH 5.0 buffer and bubbling N_2; open circles pH 5.0 buffer with sodium disulfite 1%.

3.2. Determination of RCA in the plasma and brain samples

We first tested mobile phases at various pH and with various organic solvents (methanol, acetonitrile, etc.) at different percentages, however, in order to obtain a good separation of RCA and I.S. from peaks of plasma and brain an ion-pairing (TEBA) agent had to be added. In these conditions retention times of RCA and I.S. were 21 and 24 min, respectively without interfering peaks near the retention times of RCA and I.S. were present in the chromatograms of blank or basal (time 0) plasma and brain samples. Representative chromatograms are shown in Figs. 3 and 4.

3.3. Linearity

Six standard samples between 0.5 and 100 μg/ml of RCA in plasma and between 0.5 and 10 μg/ml in brain tissue were analyzed in triplicate to determine the linearity of the assay. The ratios of the areas of the peaks of RCA vs. I.S. were linearly related to the drug concentrations in the range of concentrations

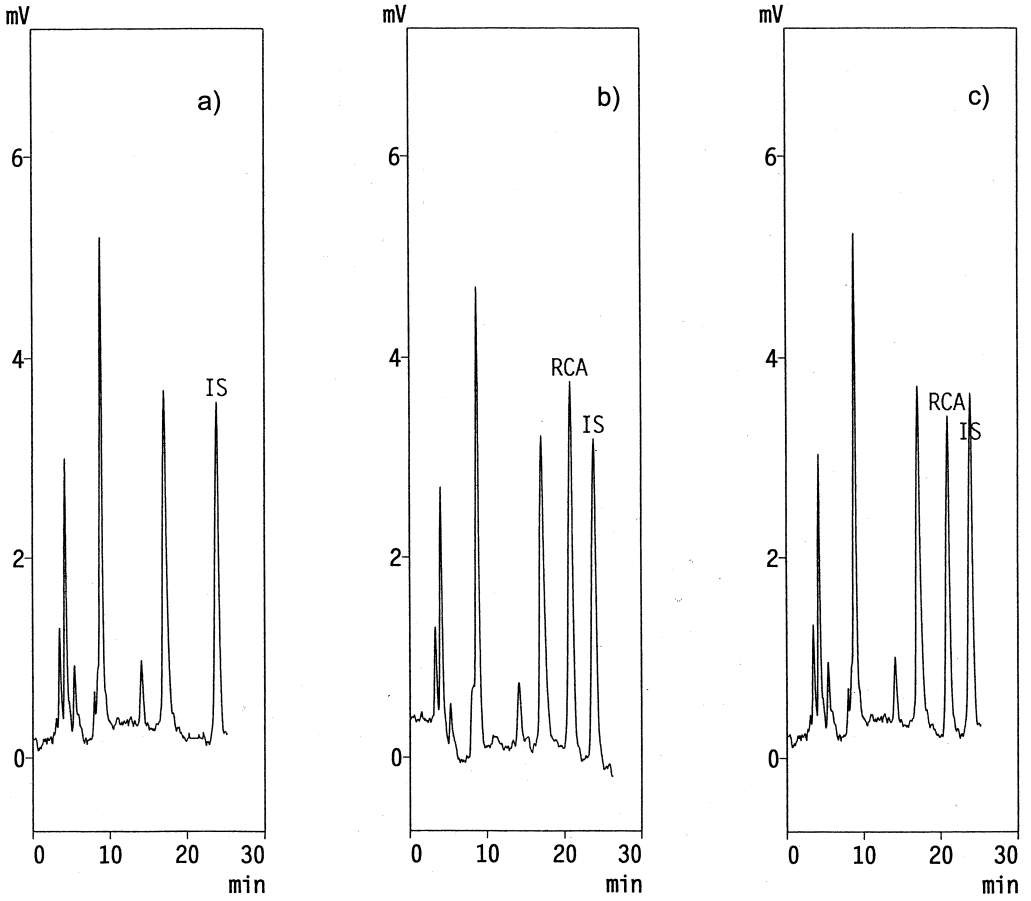

Fig. 3. (a) Plasma blank; (b) plasma standard: RCA 10 μg/ml, I.S. 10 μg/ml; and (c) plasma sample: RCA 8.72 μg/ml, I.S. 10 μg/ml.

studied both in plasma and brain tissue (plasma: $y=0.11$ ($\pm$SE 0.094)$+0.087$ ($\pm$SE 0.004)x, $r=0.99$, $P<0.001$; tissue: $y=0.108$ ($\pm$SE 0.036)$+0.094$ ($\pm$SE 0.005)x, $r=0.98$, $P<0.001$).

3.4. Accuracy and precision

Intra-day assay of RCA had a mean accuracy of 98.5% in plasma and 97.8% in brain with a mean precision (C.V.) ranging from 4.2% in plasma and 5.0% in brain. Inter-day assay of RCA had a mean accuracy of 98.6% in plasma and 97.8% in brain with a mean precision (C.V.) of 5.2% in plasma and 6.1% in brain (Table 1).

These results, therefore, validate the calibration curves used for each set of samples.

Experimental results on the quality control samples kept at -25 °C for 2 weeks guarantee the stability of the samples under our conditions (see inter-day accuracy above).

3.5. Sensitivity and recovery

The lower limit of detection of RCA in plasma or tissues (signal three times that of the blank) were 0.2 μg/ml. The lower limit of quantitation (C.V.$<$8%) of RCA in plasma or tissue was 0.5 μg/ml. The mean absolute recovery of RCA, was higher than 76.1% in plasma and 74.3% in tissue (Table 1).

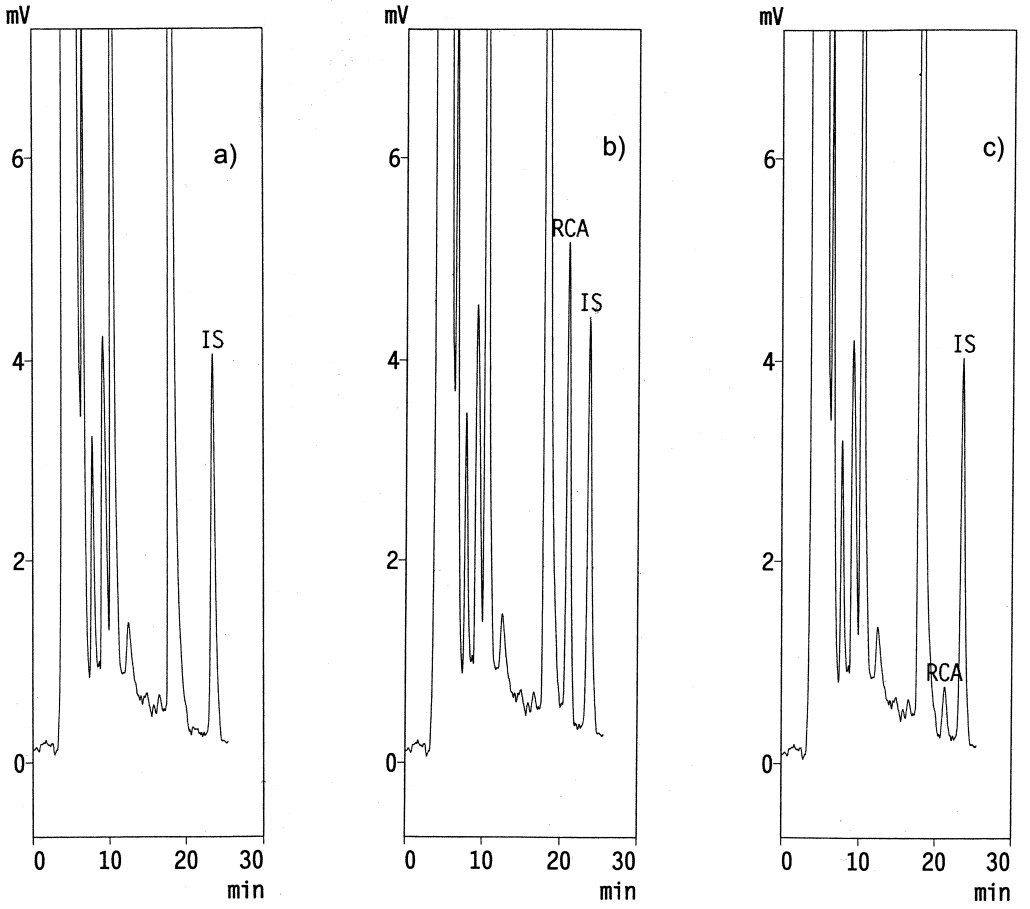

Fig. 4. (a) Brain tissue blank; (b) brain standard: RCA 10 μg/ml, I.S. 10 μg/ml, (c) brain sample: RCA 1.04 μg/ml, I.S. 10 μg/ml.

3.6. RCA concentrations in biological samples

Peak concentrations of RCA in plasma and in brain of hamsters after i.p. administration are shown in Table 2.

4. Discussion and conclusions

The study describes an analytical method to extract and quantitate a Congo-Red derivative, the sodium 3,4-diaminonaphthalene-1-sulfonate (RCA),

Table 1
Precision, accuracy and recovery for the determination of RCA in plasma and brain tissue by the described procedures

	Nominal concentrations (μg/ml or μg/g)	Intra-assay precision (C.V.%) (n=10)	Inter-assay precision (C.V.%) (n=6)	Intra-assay accuracy (%) (n=10)	Inter-assay accuracy (%) (n=6)	Recovery (%) (Mean±SD)
Plasma	1	6.2	7.8	96.7	95.4	76.1±3.5
	10	2.8	3.3	97.8	100.5	78.8±2.5
	100	3.5	4.6	101.1	99.9	79.1±2.9
Brain tissue	1	7.5	8.6	95.1	94.0	74.3±4.5
	2	4.4	5.2	99.5	97.7	78.1±4.5
	5	3.1	4.5	96.9	98.1	80.1±3.8

Table 2
Peak concentrations of RCA in plasma and brain after i.p. administration ($n=6$)

	T_{max} (min)	RCA concentrations (Mean±SD)	Brain/plasma% (Mean±SD)
Plasma (μg/ml)	15	59.9±6.6	–
Brain (μg/g)	30	1.1±0.7	1.9±0.2

in plasma and brain tissue. Both the exctraction and the chromatography were particularly difficult because of the physicochemical characteristics of the compound, i.e. its amphoteric properties due to the presence of basic (amino) and strong acidic (sulfonate) groups making the separation from the biological matrices by solvent extraction almost impossible. The chromatography of amino-sulfonated compounds and the indication for the use of ion-pairing has already been described in the literature [11–13]. Methods for the extraction of amphoteric compounds with amino and acidic groups of lower strength (e.g. aminosalycilic acid, betalactam antibiotics, etc.) from biological matrices have been reported [14,15], these methods however, are not applicable for compounds such as those assessed in our study, which were separated by using a particular type of columns for solid-phase extraction together with the use of a counter ion. In these conditions the compound was very strongly trapped by the column allowing extensive washing of the phase and thus the purification of the sample. The resulting solid-phase extraction is efficient and rapid, allowing the extraction of several plasma samples on the same day.

The second characteristics of RCA is its rapid decay by oxidation, thus an antioxidant needs to be added to each step, from collection (plasma and brain tissue) to extraction in order to prevent its degradation. In these conditions extracts and biological samples have been found to be stable over 24 h at room temperature.

The method described allows the study of the pharmacokinetics of RCA in plasma after i.p. administration. The concentrations in brain were much lower than in plasma, those at the peak time being about 2% of those found in plasma. The use of this method to determine RCA in other tissues (heart, spleen, nerves) is being investigated.

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