

## Original Research Article

# A sensitive and rapid HPLC-DAD method for the determination of 3-hydroxy-1,2-dimethyl-4-pyridone and its distribution in rats

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### Abstract

**Purpose:** To establish a sensitive and rapid method for the determination of the tissue distribution of 3-hydroxy-1,2-dimethyl-4-pyridone (L1) *in vivo*, and its plasma protein binding capacity.

**Methods:** This study optimized a reverse-phase HPLC method for specific and sensitive determination of L1 as well as its plasma and tissue distributions. The optimized method was used to determine the plasma protein-binding capacity of L1 in Wistar rats.

**Results:** A rapid, sensitive and simple HPLC-DAD method was established for studying the plasma and tissue distribution of L1. Following TI administration, its liver concentrations peaked at 60 min and 360min, followed 360 min later with peak level in the kidney (second highest). The L1 concentration was significantly lower after 360 min than after 60 min, and values of its mean binding to plasma proteins was 5.2 % at different L1 concentrations.

**Conclusion:** These results indicate that L1 is a drug with rapid-absorption and rapid-elimination that is distributed widely *in vivo* in rats. Moreover, the drug has a weak plasma protein-binding capacity.

**Keywords:** 3-Hydroxy-1,2-dimethyl-4-pyridone, Distribution, Alzheimer's disease, Therapy

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## INTRODUCTION

Aluminum is associated with chronic toxicity, with the nervous system as its main target [1]. Studies have shown that aluminum is a possible risk factor for some neurodegenerative illnesses, including Alzheimer's disease [2, 3]. 3-Hydroxy-1,2-dimethyl-4-pyridone (L1) is an orally-active iron chelator which is used for the treatment of iron overload [4]. In addition, L1 has aluminum-chelating capacity [5]. Thus, L1 may be beneficial in the prevention and treatment of

neurodegenerative illnesses through enhancement of excretion of aluminum. However, high-dose L1 may lead to toxic effects *in vivo* through chelation of essential elements. With growing significance of L1, a number of analytical methods have been established for determination of its distribution *in vivo*, but these methods lack satisfactory repeatability and convenience of use [6-8].

In the present study, a reverse-phase HPLC method for the specific and sensitive detection of

L1 was optimized and applied for the determination of plasma and tissue distributions of L1 in rats, as well as L1 plasma protein-binding capacity of L1.

## EXPERIMENTAL

### Materials and reagents

3-Hydroxy-1,2-dimethyl-4-pyridone was obtained from Aladdin Industrial Corporation; HPLC-grade methanol and acetonitrile were products of Yuwang Industrial Corporation. Sodium heptanesulfonate,  $\text{NaH}_2\text{PO}_3$  and other reagents were AR grade. Deionized water was obtained from a Milli-Q apparatus (Millipore, Bedford, MA, USA).

### Animal sub-grouping and treatments

Fifty male Wistar rats (mean weight =  $325 \pm 20$  g) were obtained from the Laboratory Animal Center, Shandong University. The experimental procedures used in this study were approved by the Institutional Animal Ethics Committee of the College of Public Health, Shandong University (approval number: 20120601). Forty rats were randomly divided into 4 groups (10 rats per group) for plasma and tissue distribution studies. The blank group was orally administered 1 mL of physiological saline, while rats in the other groups were treated with L1 at a dose of 70 mg/kg. The rats were sacrificed via decapitation after 10, 60 or 360 min.

Blood, heart, kidney, stomach, liver, spleen, lung, testis, muscle, brain and small intestine were taken out rapidly. The blood samples were anticoagulated with heparin, and the tissues were washed with physiological saline, blotted with filter paper, and made into 20 % homogenates. The other 10 rats were decapitated directly. The anticoagulated blood samples were used for determination of plasma protein binding capacity of L1. The samples were centrifuged at  $604 \times g$  for 10 min, and the resultant plasma samples were kept at  $4^\circ\text{C}$ . All tissue homogenates were preserved at  $-20^\circ\text{C}$  prior to use.

### HPLC conditions

This study was conducted using HPLC coupled to a diode array detector with a  $\text{C}_{18}$  column (5  $\mu\text{m}$ , 250 mm  $\times$  4.6 mm, Diamonsil) at  $25^\circ\text{C}$ . The mobile phase consisted of acetonitrile-phosphate buffer (pH = 3) at volume ratios of 8:92 for plasma, and 1:9 for tissue homogenates and extra-dialysate. A flow rate of 1.0 mL/min was used.

### Plasma and tissue distribution analyses

Each sample was diluted with an equal amount of acetonitrile, and centrifuged at  $6708 \times g$  for 5 min. Then, 100  $\mu\text{L}$  supernatant was taken and diluted with 400  $\mu\text{L}$  mobile phase. An aliquot (20  $\mu\text{L}$ ) of the supernatant was injected into the HPLC system after filtration with a 0.45  $\mu\text{m}$  filter.

### Plasma protein binding assay

#### Balance time analysis

Blank plasma (1 mL) was put into a dialysis bag, and placed in 10 mL of different concentrations of L1 at  $37^\circ\text{C}$ . The concentrations of drug inside ( $c_1$ ) and outside ( $c_2$ ) were determined. The plasma protein binding of L1 ( $f$ ) was calculated using equation:

$$F = (c_1 - c_2) / c_1 \times 100 \dots\dots\dots (1)$$

#### Absorption of dialysis bag

In this measurement, 1 mL ( $V_1$ ) of phosphate buffer (pH = 7.4) was put into a dialysis bag, and placed in 10 mL ( $V_2$ ) of different concentrations ( $c_3$ ) of L1 at  $37^\circ\text{C}$  for 4 h. The concentrations of the extra-dialysate ( $c_4$ ) were determined. The absorption was calculated as shown in Equation 2:

$$X = \{[c_3 \times V_2 - c_4 \times (V_1 + V_2)] / (c_3 \times V_2)\} \times 100 \dots\dots\dots (2)$$

### Plasma protein binding of L1 in samples

The intra-dialysate and extra-dialysate samples were reconstituted with 100  $\mu\text{L}$  acetonitrile, and centrifuged at  $6708 \times g$  for 5 min. Then, 100  $\mu\text{L}$  supernatant was mixed with 400  $\mu\text{L}$  mobile phase, and an aliquot (20  $\mu\text{L}$ ) of the supernatant was injected into the HPLC system after filtration through a 0.45  $\mu\text{m}$  filter.

### Statistical analysis

Data are expressed as mean  $\pm$  standard deviation (SD). Statistical analysis was done using Student's *t*-tests. Statistical significance of difference was fixed at  $p < 0.05$ .

## RESULTS

### Optimization of HPLC-DAD

#### Detection wavelength

A standard solution of L1 was scanned between 190 nm and 400 nm in an Ultraviolet-Visible Spectrophotometer. The scanning spectrum of

L1 is shown in Figure 1. There were strong UV absorptions at wavelengths of 202, 216 and 278 nm.

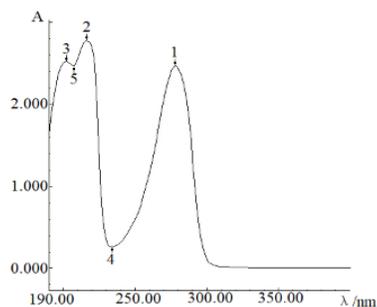


Figure 1: UV spectra of L1

**Method validation**

The chromatograms of the blank plasma, tissue homogenates and extra-dialysate were obtained using the optimized HPLC method (Figure 2). Blank plasma, tissue homogenates and extra-dialysate with L1 standard were analyzed (Figure 3). The chromatograms for plasma, tissue homogenates and extra-dialysate of samples are shown in Figure 4. It is apparent that the endogenous substances in plasma, tissue homogenates and extra-dialysate did not interfere with the peak of L1. Thus, the optimized method is specific for the measurement of L1.

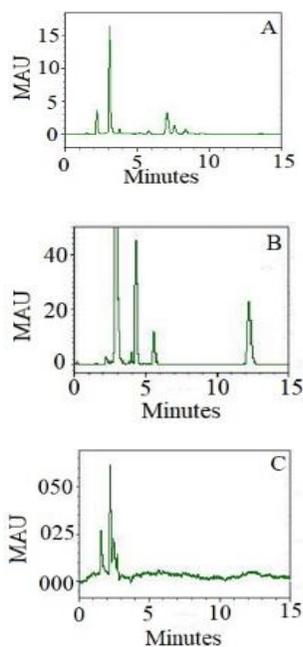


Figure 2: HPLC chromatograms of blank groups. A: plasma blank; B: tissue homogenate blank; C: extra-dialysate blank

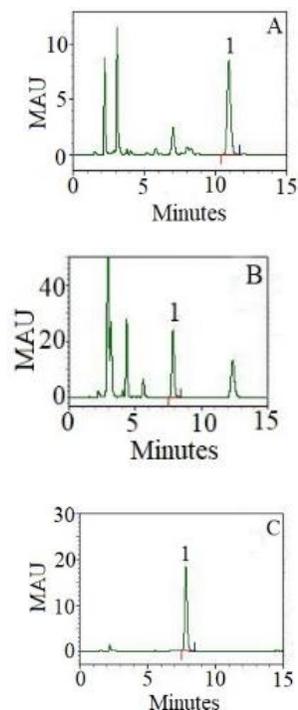


Figure 3: HPLC chromatograms of L1 in the blank plasma, tissue homogenate blank and extra-dialysate blank. A: plasma blank with L1; B: tissue homogenate blank with L1; C: extra-dialysate blank with L1

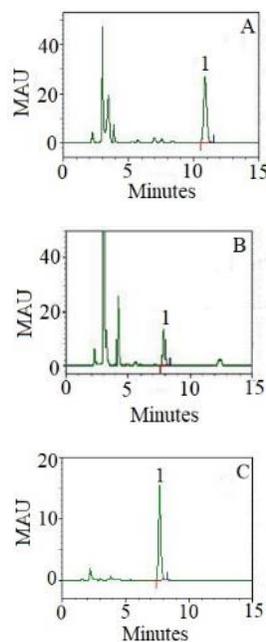


Figure 4: HPLC chromatograms of L1 in rat plasma, tissues and dialysate. A: plasma sample; B: tissue homogenate; C: extra-dialysate

**Standard curve**

When L1 concentration was in the range of 1-100 mg/L in plasma or 0.1-100 mg/L in tissue

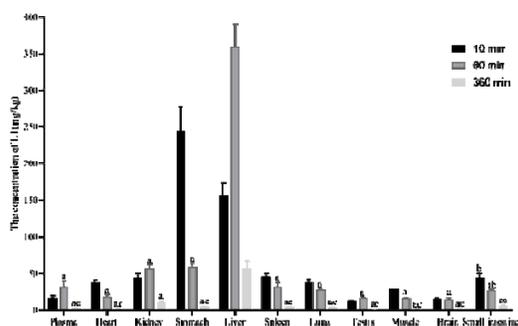
homogenate and extra-dialysate, peak area and concentration showed good linear relationship, with the regression equations:  $y = 29952.49x - 4137$  ( $r = 0.99974$ ), and  $y = 31143.78x - 2663$  ( $r = 0.99983$ ), respectively. The limits of detection were 6.1 and 5.9  $\mu\text{g/L}$ , respectively.

### Recovery and precision

Serial concentrations of standard solution of L1 (5, 40 and 80 mg/L were added to the plasma blank. The concentration of L1 was determined 5 times repeatedly for each solution. The tissue homogenate blank and extra-dialysate blank were treated in the same way. In plasma, the recoveries were in the range of 99.04 - 100.34% at low, middle and high concentrations, while in tissue homogenates and extra-dialysate, the recoveries were in the range of 95.93 - 105.22%. The coefficients of variation at all concentrations were less than 4.1%.

### Distribution of L1 in the rats

As shown in Figure 5, 10 min after intragastric administration of L1, it was detected in plasma and all tissues, with the highest level in the stomach. After 60 min, the liver content of L1 (247.3 mg/kg) was significantly higher than that in other tissues. After 360 min, liver L1 content (57.3 mg/kg) was significantly higher than that in other tissues, followed by the kidney (12.1mg/kg). The plasma and tissue concentrations of L1 at 360 min were significantly lower than those at 60.

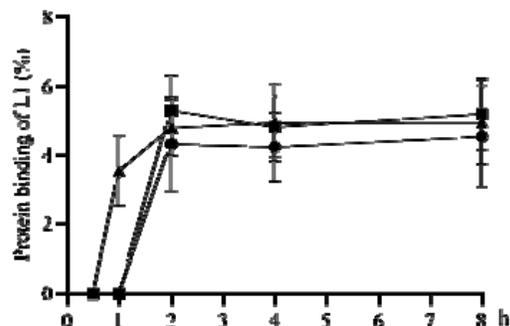


**Figure 5:** L1 distribution in tissues at a dose of 70 mg/kg at 10 min, 1h and 6 h. a:  $p < 0.001$ , compared with the liver concentration of L1 in the same group, <sup>b</sup> $p < 0.001$ , compared with the stomach concentration of L1 in the same group; <sup>c</sup> $p < 0.001$ , compared with that of kidney in the same group

### Plasma protein binding

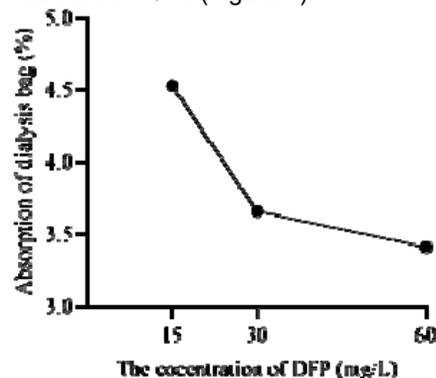
It took at least 2 h to reach dialysis equilibrium for L1 at 37 °C under the experimental conditions (Figure 6). After dialysis equilibrium, the average

percentage of L1 bound to plasma proteins ranged from 4.1 to 5.2%. There were no significant differences in the capacities of different concentrations of L1 to bind to plasma proteins ( $p > 0.05$ ).



**Figure 6:** Protein binding of various L1 concentrations ( $n = 5$ . Key: ● = 15 mg/L, ■ = 30 mg/L, ▲ = 60 mg/L)

The mean adsorption of L1 to the dialysis bag was below 4.6% (Figure 7).



**Figure 7:** Adsorption of L1 to dialysis bag

## DISCUSSION

As shown in the results, L1 had strong UV absorption at wavelengths of 202 nm, 216 nm and 278 nm. Since the mobile phase and the impurity in plasma interfere with the detection of L1 at short wavelengths, 278 nm was chosen as the detection wavelength in order to get better sensitivity and accuracy. To improve the peak symmetry and resolution of L1 from impurities, the chromatographic behaviors of different mobile phases i.e. trifluoroacetic acid-acetonitrile [6], trifluoroacetic acid-methanol, PBS-acetonitrile [9], and PBS-methanol [10] were investigated. Furthermore, to eliminate tailing of peak, sodium heptanesulfonate was added. After many trials, a mobile phase containing acetonitrile-PBS (0.05 mol/L  $\text{NaH}_2\text{PO}_3$ , 5 mmol/L

sodium heptanesulfonate and 2 mmol/L EDTA, pH = 3 (8:92 or 1:9, v/v) was set up. The results of validation and recovery and precision experiments showed that a rapid, sensitive, and simple method was successfully established for the determination of L1 which is suitable for use in studying the distribution and plasma protein binding capacity of L1.

Ten minutes after administration, L1 was detected in plasma and all tissues, including brain and testis, which means that L1 could cross the blood-brain and the testicular barrier. 3-Hydroxy-1,2-dimethyl-4-pyridone was used to clear the aluminum-overload in the brain, as suggested in a previous study [5]. Drug level in the stomach was highest at 10 min after dosing, which may be due to the fact that gastric emptying time in rats exceeds 10 min. In the first 60 min, the stomach concentration of L1 was far higher than that in the small intestine. However, 60 min later, the stomach concentration of L1 decreased rapidly, but L1 level in the small intestine did not increase significantly, which indicated that L1 was rapidly absorbed mainly in the stomach. The content of L1 in liver was highest except in the first 10 min, indicating that the liver may have a certain affinity for L1. It was previously reported that L1 goes mainly to the liver after administration, with only very small amount metabolized via glucopyranoside acidification [11]. The content of the drug was highest in the liver 360 min after administration, indicating that its elimination was slow. Thus, L1 might accumulate in the liver after administration. This needs further investigations.

Highly aerobic tissues such as liver, spleen, heart, lung and kidney had higher concentrations of L1, while those with poor blood oxygen content had lower L1 concentrations. The wide distribution of L1 might be related to its small molecular weight (139.15), high lipo-solubility and easy passage through the cell membrane via passive diffusion. 3-Hydroxy-1,2-dimethyl-4-pyridone is filtered and excreted mainly in the kidney [12,13]. Most L1 is reabsorbed into the blood in the tubular cells through passive diffusion [14], while the remaining L1 is excreted in the urine. In this study, at 360 min after its administration, the kidney L1 content was next to that of the liver, but was below 1/5<sup>th</sup> of the corresponding concentration in liver. It is difficult for L1 to accumulate in rats, as evidenced by its short elimination half-life and quick clearance [15].

3-Hydroxy-1,2-dimethyl-4-pyridone exists partly in the free form in plasma, while some are bound to plasma proteins. The binding of drugs to

plasma proteins directly or indirectly influences their transportation and pharmacological effects [16]. Usually, there is a dynamic equilibrium between the bound and free forms of a drug. The plasma protein-binding capacities of drugs are measured using equilibrium dialysis method [17], ultrafiltration [18], gel electrophoresis and microdialysis [19]; ultracentrifugation, and spectroscopic methods. The equilibrium dialysis method is simple, economical and devoid of interference. Perchloric acid was used to detect protein leakage after dialysis. The adsorption of L1 to the dialysis bag was minimal, indicating that it had no significant influence on the results. Plasma protein binding was obtained from the calculation of drug concentrations inside and outside the dialysis bag. There were no significant differences in percentage plasma protein binding among the low, middle and high concentrations of L1. Thus, the plasma protein binding of L1 was not concentration-dependent. Combination of drugs and plasma affects the action time of the drugs. The action time is shortened if the percentage plasma protein binding is low. In this study, L1 displayed pharmacodynamics mainly in the free form and so could be minimally competitive with other medicines.

## CONCLUSION

A rapid, accurate, sensitive and reproducible HPLC-DAD method for the determination of 3-hydroxy-1,2-dimethyl-4-pyridone has been established in this study. The results suggest that 3-hydroxy-1,2-dimethyl-4-pyridone is a rapid-absorption and rapid-elimination drug which is distributed widely *in vivo* in rats. Moreover, the drug has a low plasma protein-binding capacity.

## DECLARATIONS

### Acknowledgement

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### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. The work

was originally designed by Mengdi Chen, Ping Liu, Xuejiao Zhang, Shuling Li and Qiongyao Zhang, and the studies were carried out them. The first two authors wrote this manuscript. All authors read the manuscript before it was submitted for publication.

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