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Preparation, characterization and *in vivo* distribution of solid lipid nanoparticles loaded with cisplatin

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An emulsification dispersion-ultrasonication method was employed to prepare solid lipid nanoparticles (SLN) loaded with cisplatin. The conventional antitumor drug cisplatin (CDDP) was incorporated into SLN to obtain a targeted and less toxic drug delivery system. The CDDP-SLNs were spherical and uniform in transmission electron microscopy (TEM) photography. The mean particle size and zeta potential were 121 ± 15 nm, and −46.4 ± 10.3 mV, respectively. Also, a novel cation exchange resin method was adopted to investigate the encapsulation efficiency (EE%) of the SLN. This method is based on the principle of cation exchange between drugs and resins, and the EE% of the optimal formulation was 82.3%. The *in vitro* release profile revealed that CDDP was released from SLN efficiently and completely in normal saline (NS) compared with other release media. A pre-column derivatization HPLC method was established for *in vivo* assay of cisplatin. A tissue distribution study was conducted in male rats after iv administration of 8 mg · mL^{-1} CDDP-SLN and cisplatin NS, and it was found that CDDP-SLN has a targeted effect to the liver as well as a low concentration in the kidney in rats. These results indicated that emulsification dispersion-ultrasonication is a simple, easy, available and effective method for preparing CDDP-SLN, and the cation exchange resin method is a feasible and suitable method to evaluate the EE% of CDDP-SLN. CDDP-SLN prepared by this method was proved to be a targeted and less toxic drug delivery system.

1. Introduction

During the last decade, solid lipid nanoparticles (SLN) have been developed as a promising carrier system for controlled drug delivery. As a colloidal drug carrier, SLN are particles of solid lipid with a mean diameter between approximately 50 and 1000 nm (Müller et al. 2000; Wissing et al. 2004). These solid lipids can avoid toxic residues, which are common when polymeric nanoparticles are used. Compared with ordinary particulate carriers, SLN have many advantages, such as good biocompatibility due to the use of physiological lipids (Müller et al. 1996), high bioavailability and efficient targeting (Mehnert et al. 2001). They are also suitable for large-scale production by high pressure homogenization. Therefore, research into SLN for targeted drug delivery systems is currently attracting increasing interest.

Cisplatin (*cis*-diamminedichloro-platinum, CDDP) was the first platinum drug to be used clinically. With a wide spectrum of antitumor activities, cisplatin is widely used in the treatment of solid malign cancers, such as thyroid cancers, lung cancers, head and neck cancers and genitourinary cancers (Giaccone 2000). However, severe toxic side effects including nephrotoxicity, peripheral neuropathy and ototoxicity have inhibited its clinical use to a great extent. Several dosage forms such as liposomes, microspheres and core-surface-cross linked nanoparticles have been developed (Xiao et al. 2003; Fujiyama et al. 2003; Xu et al. 2006). Nevertheless, severe toxicity remains and targeted delivery has not yet been achieved.

In our study, cisplatin was embedded into the bilayer lipid matrix of SLN which prevents drugs from degradation and significantly reduces the danger of acute and chronic toxicity (Mehnert et al. 2001). We developed an emulsification dispersion-ultrasonication method for the preparation of cisplatin-SLN (CDDP-SLN). With this method, the nanoparticles are obtained by two steps: the formation of an O/W emulsion (aqueous phase including Pluronic F68, combined with a lipid phase containing glyceryl monostearate (GMS), Tween 80 and soybean phospholipid), followed by dispersion of the initial emulsion in cold water and ultrasonic treatment to obtain a fine particle size. The properties of CDDP-SLN, including morphology, particle size, zeta potential, entrapment efficiency (EE%) and *in vitro* release behaviour were studied in detail. In addition, the tissue distribution of CDDP-SLN and a control formulation in rats was studied to investigate its *in vivo* characteristics.

2. Investigation, results and discussion

CDDP-SLN was prepared using an emulsification dispersion-ultrasonication method. Before preparation, SP was dispersed in distilled water (5% w/v), and the drug was

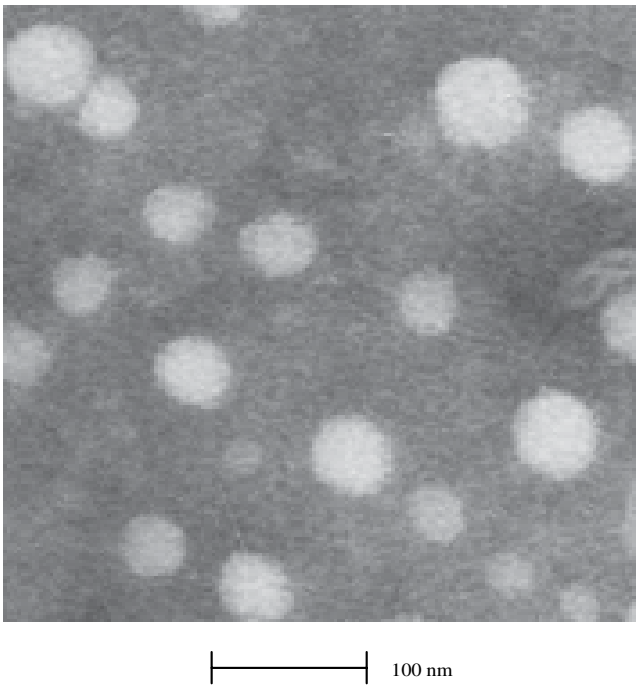

Fig. 1: TEM photograph of CDDP-SLN (× 40,000)

dissolved in DMF solution before addition. The former method was used in order to enhance the emulsification and aqueous-dispersibility of SP, as previously reported (Giri et al. 2005), and the latter was due to the instability of cisplatin in water.

Formulation factors have to be carefully considered. The type and quantity of SP had significant effects on the appearance and stability of SLN. According to our preliminary experiments, the appropriate concentration range of SP was 5–10 mg · mL^{-1}, as below this range the EE% was too low to control the quality and concentrations above resulted in reduced emulsification. Furthermore, the amount of Tween 80 was limited to 0.4% (w/v, 5 mg · mL^{-1} in 20 ml dispersion), because a higher concentration leads to haemolysis following intravenous injection. The influence of GMS on drug loading capacity was also marked, the optimal concentration of GMS being 5 mg · mL^{-1}, while Pluronic F68 plays a relative minor role as far as the properties of the formulation are concerned.

In addition, the preparation conditions warrant some discussion. On account of the thermolability and light-sensitivity of CDDP (Tang et al. 2000), the optimum temperature was below 60 °C, and the whole preparation was protected from intensive light. It has been reported that CDDP decomposes easily under high ultrasonic power (Macka et al. 1994), so the power and duration of ultrasonication needed to be carefully controlled and thus 300 W and 3 min were the optimal choices.

TEM photography showed that the CDDP-SLN particles had subsphaeroidal and uniform shapes (see Fig. 1). The mean diameter was in the range of about 40–100 nm. The morphology of the CDDP-SLN indicated that CDDP-SLN were successfully obtained by the above method.

The average diameter of CDDP-SLN obtained was 121 ± 15 nm (n = 3), and the particle size distribution was as shown in Fig. 2(a). The SLN used for drug loading exhibited a small particle size and a narrow size distribution. Therefore, transparent, stable and highly dispersible nanometric dispersions could be prepared using this method.

Measurement of zeta potential is required to assess the properties of the charged particles. In general, highly charged particles are less likely to aggregate and flocculate' due to electrical repulsion (Müller et al. 2000), and so storage stability can be increased. The mean zeta potential of CDDP-SLN was −46.4 ± 10.3 mV (n = 3), with a zeta potential distribution as shown in Fig. 2(b). As a result, a highly stable SLN dispersion was obtained.

We employed a cation exchange resin method to measure the EE% of CDDP-SLN. The principle of this method is based on the exchange of positive charges between cisplatin and sodium ions held on the resin (Wang et al. 2002). As we know, cisplatin forms a hydrolysis product in water, since the dichloro groups are easily displaced by the diaqua groups, and, therefore, the charge of cisplatin in aqueous dispersions is mostly positive (Yotsuyanagi et al. 2002). As for SLN suspensions, the free cisplatin molecules are positively charged while the charge of embedded drug molecules remains neutral. As a result, the amount of cisplatin entrapped in lipid materials may be calculated.

It was clearly confirmed that CDDP in solution and blank SLN were easily adsorbed by resins. As a result, the

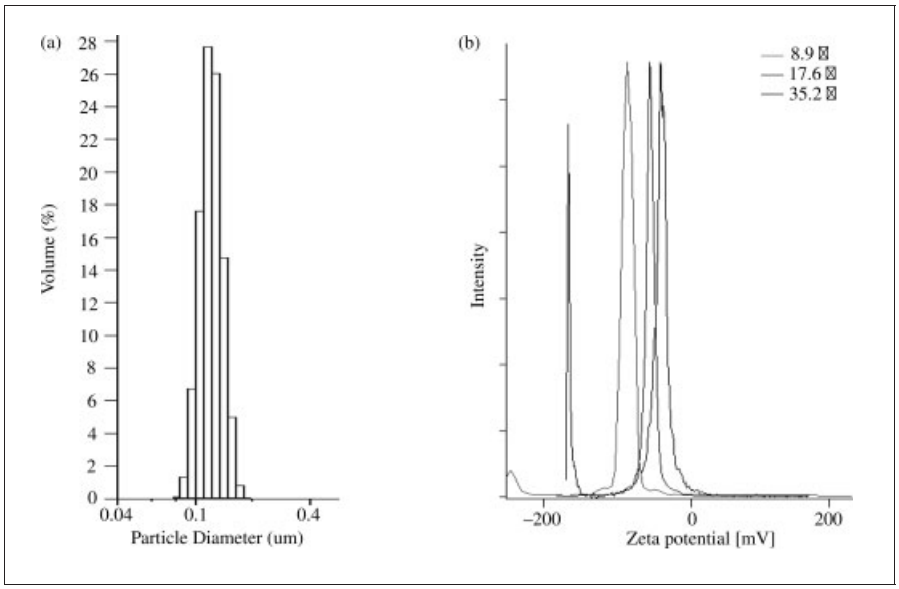

Fig. 2:
(a) Particle size distribution. (b) Zeta potential distribution of CDDP-SLN (n = 3)

Table 1: Adsorption of CDDP in solution by cation exchange resin (n = 3)

C ($mg \cdot mL^{-1}$)	0.05	0.1	0.2
A_{after}	1028.7	1610.4	2820.3
A_{before}	19972	38576	75430
Resolution (%)	94.8	95.8	96.3

Table 2: Adsorption of CDDP with blank SLN by cation exchange resin (n = 3)

C ($mg \cdot mL^{-1}$)	0.05	0.1	0.2
A_{after}	862.0	1620.5	2172.3
A_{before}	18585	37683	71559
Resolution (%)	95.4	95.7	97.0

Table 3: Recovery of blank SLN from cation exchange resin (n = 3)

V (mL)	1.0	2.0	3.0
A_{before}	0.259	0.523	0.788
A_{after}	0.251	0.512	0.779
Recovery (%)	103.2	102.1	101.2

method used to measure the EE% of CDDP-SLN was feasible and reasonable. The statistical data are given in Tables 1, 2 and 3.

The drug loading capacity of the lipid depends on a number of factors including its lipophilicity, solubility in solid lipid melt, emulsifiers used during the preparation, and ratio of drug to lipid carrier. The positively charged hydrated cisplatin is attracted to the negatively charged phospholipid (Velinova et al. 2004), so it was predicted that the EE% would be higher than that of particle carriers without phospholipid. The EE% of several formulations are shown in Table 1. It was clear that the optimal one was formulation D.

However, we can see that the EE% values were relatively lower than previously predicted. This might be due to the following reasons: The solubility of cisplatin in water is 8.43^{-3} mol $\cdot$ L^{-1} (0.253 g per 100 g of water) at 25 °C, which is defined as slightly soluble in water (ChP, 2005, examples, X) (Yotsuyanagi et al. 2002). As a result, some of the dispersed cisplatin would dissolve in to solution instead of being entrapped in lipid when incorporated into the formulation, even though the electrostatic attraction force was very strong. In addition, the solubilization of the emulsifiers is an important factor leading to a reduced EE% and, the addition of Tween 80 was a key factor. We can conclude from Table 4 that SLNs with a higher concentration of Tween 80 exhibited a relatively lower EE% than those with a lower concentration.

The *in vitro* release profiles of cisplatin from CDDP-SLN in three dissolution media are shown in Fig. 3. In all cases, loaded cisplatin was released within 36 h and no conspicuous burst release was observed in any medium. The release rates in the two PBS were similar with a slight increase at higher pH. It is obvious that the release of cisplatin from SLN in normal saline was faster than in PBS. Also, the release of CDDP-SLN in NS reached 64% of the total, which was much higher than in PBS. These results could be attributed to the high stability and solubility of cisplatin in NS. As stated above, cisplatin is easily hydrolyzed in water because of the low concentration of chlorine ions and so is relatively stable in NS. Therefore, the free drug could permeate into NS solution at random, and drug loaded in SLN could be easily released into the receiving phase through the membrane, due to its high solubility in the release media.

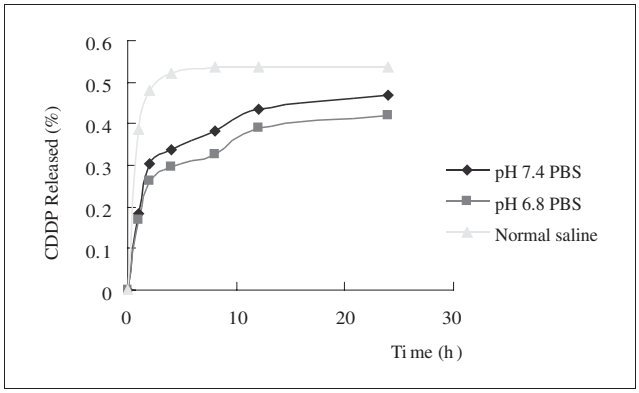

Fig. 3: In vitro release profiles of CDDP from SLN in three different dissolution media at 37 ± 1 °C: Phosphate buffer, pH 7.4 (a). Phosphate buffer, pH 6.8 (b). Normal saline (c). (n = 3)

In our work, a rapid, accuracy, sensitive and precise pre-column derivatization HPLC method was established for the determination of cisplatin. Although several methods for the assay of cisplatin plasma concentration using atomic absorption spectroscopy have been reported (Li et al. 1999; Tamura et al. 2002), cisplatin analysis in plasma and organs can be subject to variability and indeterminateness. The use of a method of pre-column derivatization with DDTC using HPLC for monitoring cisplatin has been reported, with ultrafiltered plasma used to remove proteins (Auey et al. 1995; Flores et al. 2005). The $Pt(DDTC)_2$ complex was derivatized by the reaction of DDTC and cisplatin, which could be extracted with chloroform and detected at 254 nm.

In our study, we employed a pre-column derivatization HPLC method without ultrafiltration of plasma and homogenates. As we know, cisplatin tends to form protein binding products in plasma, which influence the assay of its total amount. Acetic ether was added to plasma and homogenates in order to release cisplatin bound to proteins, so that cisplatin existed as free drug which was available for determination. The acetic ether was then easily volatilized under nitrogen.

Table 4: EE% of several formulations of CDDP-SLN (n = 3)

Formulation	GMS ($mg \cdot ml^{-1}$)	SP ($mg \cdot ml^{-1}$)	F-68 ($mg \cdot ml^{-1}$)	Tween 80 ($mg \cdot ml^{-1}$)	EE (%)
A	2.5	5.0	5.0	2.5	79.1
B	2.5	5.0	10.0	5.0	52.9
C	5.0	5.0	10.0	2.5	80.5
D	5.0	10.0	5.0	2.5	82.3
E	10.0	10.0	5.0	5.0	58.1

Table 5: Concentration of cisplatin NS and CDDP-SLN in rat after iv administration (n = 6)

Samples	Concentration ($\mu g \cdot g^{-1}$) (SLN/N.S.)			
	10 min	20 min	40 min	60 min
Plasma	39.8/28.4	23.9/42.2	19.8/27.3	10.2/17.1
Liver	66.7/11.1	74.3/12.4	62.1/6.0	35.3/2.1
Kidney	11.8/12.7	16.9/19.5	14.2/9.5	8.9/10.0
Lung	2.2/4.0	5.8/1.7	7.3/0.4	1.3/0.3

Table 5 shows the concentration of CDDP-SLN and cisplatin NS in plasma, liver, kidney and lung, respectively. It can be clearly seen that in rats CDDP-SLN was mostly concentrated in the liver, while the concentrations of cisplatin in lung and kidney were much lower than in liver and plasma, with, it should stressed, the lowest in the lung. Furthermore, compared with cisplatin NS, the solid lipid matrix plays an important role in protecting the drug from degradation in plasma and organ cells. It is obvious that CDDP-SLN showed targeted delivery to the liver and conspicuously decreased nephrotoxicity. In other words, CDDP-SLN was proved to be a targeted and less toxic drug delivery system.

3. Experimental

3.1. Materials

Cisplatin was purchased from Suzhou Leader Chemical Co.Ltd. Nickel chloride (obtained from the No.1 Kaiyuan Chemical Agent Co.Ltd) was used as internal standard. Glyceryl monostearate (GMS, obtained from Changsha Organic Chemical Co.Ltd.) was used as the lipid carrier. Soybean phospholipid (SP, for injection) was supplied by Shanghai Taiwei Pharmaceutical Industry Co.Ltd. Tween 80 was obtained from Tianjin Bodi Chemical Co.Ltd. and Pluronic F68 was kindly donated by BASF. Cation exchange resins were obtained from Shanghai Resin Co.Ltd. Dimethylformamide (DMF) and other reagents were of analytical reagent grade.

3.2. Preparation of CDDP-SLN

GMS (100 mg) was dissolved in absolute alcohol (5 mL) and heated to 60 °C in a water bath, then SP (200 mg) and Tween 80 (50 mg) were added to the above solution as the oil phase. The aqueous phase consisted of F68(100 mg) and distilled water (5 mL), also heated to 60 °C. A few drops of cisplatin DMF solution (equal to 20 mg CDDP) was poured into the oil phase under vigorous agitation, then the oil phase was added to the aqueous phase, dropwise under magnetic stirring (1000 rpm, DF-101S; Gongyi Yuhua Equ.Inst., China) at constant temperature for 30 min. During this process, the original emulsion was formed and the absolute alcohol was completely evaporated. Then the suspension was dispersed in 2–5 °C water (the ratio of suspension to water 1 : 4, v/v) under mechanical stirring. The transparent phase was then subjected to ultrasonic treatment at 300 W for 5 min using a high-intensity probe ultrasonicator (JY92–2D; Ningbo Xinzhi Equ.Inst., China). The resultant CDDP-SLN suspensions were filtered through a 0.45 μm micropore filter in order to remove the residual metal formed by the ultrasonication. The final concentration of the CDDP-SLN formulation was 1 mg · mL^{-1}. All the operations were carried without exposure to intense light.

3.3. Transmission electron microscopy (TEM)

The morphology of CDDP-SLN was examined by TEM (JEM-1200EX, JEOL). The samples were appropriately diluted with distilled water and stained with 2% (w/v) phosphotungstic acid for observation.

3.4. Particle size and zeta potential measurement

The mean size of SLNs in the suspension was determined by dynamic light scattering (DLS) using a laser particle sizer (LS630, Beckman Coulter). The particle size data were evaluated as the volume distribution. The zeta potential was measured by a zeta potential analyzer (Delsa 440SX; Beckman Coulter) at 37 °C. Each sample was diluted with distilled water until the appropriate concentration of particles was achieved, and each sample was measured in triplicate.

3.5. Encapsulation efficiency (EE%) determination

3.5.1. Identification experiments

The encapsulation efficiency (EE%) of CDDP was determined by a cation exchange resin method. In order to assess the feasibility of this method, some experiments were carried out.

3.5.1.1. Adsorption of CDDP in solution by cation exchange resin

An 1 mg · mL^{-1} DMF solution of cisplatin was prepared as a stock solution. The control solutions were prepared as follows: 0.5 mL, 1 mL and 2 mL portions of stock solution were pipetted into 10 mL volumetric flasks, respectively, then 2 mL distilled water was added to each flask and the volume made up with DMF. Other 0.5 mL, 1 mL and 2 mL solutions were introduced at the top of the column, which was prepared by filling a 5 mL syringe with an appropriate amount of cation exchange resin, then 2 mL distilled water was added and the volume measured. The above two solutions were shaken well to achieve an equilibrium and then filtered through a 0.45 μm membrane. They were then analyzed by HPLC (n = 3) using a delivery pump (LC-5P, Dalian Johnsson Separation Sci & Tech.Co.), and an UV-VIS detector (LC-10UV, Dalian Johnsson Separation Sci & Tech.Co.). The calibration curve of the peak area (A) versus concentration of CDDP (C) was $y = 432912x + 1544.6$, and the linear range was 0.05–0.5 mg · mL^{-1} ($R^2 = 0.9997$, where y = A and x = C). The resolution of the cation exchange resin was computed using Eq. (1).

$$\text{Resolution } (\%) = 1 - A_{after}/A_{before} \times 100\% \quad (1)$$

A_{after}, A_{before}: the peak area of the CDDP solution after and before passing through the cation exchange resin, respectively.
Chromatographic conditions: The column was a Lichrospher-NH_2 (250 mm × 4.6 mm, 5 μm), the mobile phase consisted of ethyl acetate: methanol: DMF: purified water (25 : 16 : 5 : 5, volume ratio). The flow rate and detection wavelength were 1.0 mL · min^{-1} and 310 nm, respectively. Detection was carried out at room temperature.

3.5.1.2. Adsorption of CDDP with blank SLN by the cation exchange resin

First, 10.0 mg CDDP was added to 10 mL blank SLN, so that the concentration of the drug suspension was 1 mg · mL^{-1}. The following procedures were carried out as described in Section 4.5.1.1. The control and sample solutions were prepared as above, and the resultant resolution was also calculated as before.

3.5.1.3. Recovery of blank SLN from the cation exchange resin

An UV-VIS detector (WFZ800-D_2, Secondary Optical Instrument Co.Ltd, Beijing) was used to measure the absorbance (A) of blank SLN, at a wavelength of 450 nm. The procedures were as described in Section 3.5.1.1 except that the volumes of SLN were 1, 2 and 3 mL, respectively. The sample was measured against distilled water as the control, and the recovery was calculated from Eq. (2).

$$\text{Recovery } (\%) = A_1/A_2 \times 100\% \quad (2)$$

A_1, A_2: Absorption of blank SLN before and after passing through the cation exchange resin, respectively.

3.5.2. Assay of EE%

The assay was carried out as described above. In particular, the volumes of CDDP-SLN suspension and distilled water were 0.5 mL and 2 mL, respectively. The concentrations of CDDP incorporated in SLN (C_1) and total drug (C_2) were assayed by HPLC. Equation 3 was used to calculate EE%.

$$EE(\%) = C_1/C_2 \times 100\% \quad (3)$$

3.6. In vitro release study

Drug release from CDDP-SLN was determined in phosphate buffer solution (pH 7.4 and 6.8 PBS) and NS, respectively, using the dialysis bag method. The dialysis bag retains SLN and allows the free drug to pass into the release medium with a molecular weight cut-off of 14000. After soaking in double-distilled water for 12 h, the dialysis bag was ready to use. Then, 3 mL of CDDP-SLN dispersion (1 mg · mL^{-1}) was transferred to the bag and the two ends fixed with clips, and the bags were placed in a conical flask containing 30 mL of receiving medium. The conical flasks were placed in a thermostatic shaker (SHA-B, Constant-Temperature Shaker, China) at 37 ± 1 °C and 100 strokes · min^{-1}. At predetermined time intervals, the medium in the flask was completely removed and 30 mL fresh medium was added to maintain the sink condition. The CDDP content of the filtrate was determined by HPLC (n = 3).

3.7. Tissue distribution studies in rats

3.7.1. Sample preparation

Animal experiments were carried out in accordance with the requirements of the National Act on the use of experimental animals (People's Republic of China). Twenty-four male Wistar rats (250 ± 20 g, provided by China Medical University Animals Center) were used for our studies. All animals were fasted for 12 h before the experiments but had free access to water. The rats were randomly divided into two groups: CDDP-SLN administration and cisplatin NS as control. A dose of 8 mg · kg^{-1} cisplatin was injected via the caudal vein and the animals were subsequently sacrificed at 10, 20, 40 and 60 min. Whole blood was then collected in heparinized tubes and immediately centrifuged to obtain plasma. Liver, kidneys and lung were also collected, cleaned with NS and then weighed. After adding known amounts of NS, the organs were ground to give homogenates. The plasma and homogenates were stored at −20 °C for analysis.

3.7.2. Extraction procedure

The concentration of CDDP in plasma and homogenates was determined by pre-column derivatization with diethyldithiocarbamate (DDTC, Augey at al. 1995; Flores et al. 2005). DDTC (10%) was extemporaneously prepared by dilution in 0.1 M sodium hydroxide aqueous solution. First, the plasma (or homogenate) sample (100 μL) was placed into a centrifuge tube and acetic ether (400 μL) added, then evaporated to dryness under nitrogen. The residue was dissolved in NS (100 μL), then 25 μL internal standard (200 mg · mL^{-1} nickel chloride NS) and 100 μL DDTC in NaOH solution were added, and samples were incubated in a 37 °C water bath for 30 min and then chilled. Chloroform (400 μL) was added and vortexed at maximum speed for 2 min on a Vortexer (XK96-A, Jiangyan Xinkang Medical Equipment Co,Ltd.). The two layers were separated by centrifugation at 10,000 rpm for 10 min. The chloroform was extracted to another tube, then evaporated to dryness again and the residue was dissolved in chloroform (100 μL). 10 μL was injected into the column.

Chromatographic conditions: The column was a Century SIL C_{18} (200 mm × 4.6 mm, 5 μm), the mobile phase consisted of methanol : purified water : acetonitrile (50 : 25 : 25, volume ratio). The flow rate and detection wavelength were 1.5 mL · min^{-1} and 254 nm, respectively. Detection was carried out at room temperature.

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