Role of sodium malate in the inhibitory effect of Juzen-taiho-to against cisplatin-induced toxic side effect

Harumi UEDA, Kiyoshi SUGIYAMA,* Yasuhiro KAJIMA and Masami YOKOTA

University of Shizuoka, School of Pharmaceutical Sciences

(*Received June 3, 1997. Accepted August 11, 1997.*)

Abstract

We investigated the role of sodium malate, an active constituent of the Kampo medicine Juzen-taiho-to (十全大補湯), in inhibiting the toxicity of cisplatin. The sodium malate content of Juzen-taiho-to and its constituent crude drugs was determined by HPLC. The content of sodium malate in Juzen-taiho-to was 0.98%, and sodium malate was detected in all of the constituent crude drugs, especially *Angelicae Radix* (23.1 %) and *Ginseng Radix* (14.8 %). The inhibitory effect of sodium malate on cisplatin-induced nephrotoxicity was assessed. The ED_{50} of sodium malate (p.o.) against the nephrotoxicity was 0.4 mg/kg, which was about 65 times and 500 times higher in the activity than that of *Angelicae Radix* and Juzen-taiho-to, respectively. From the content and the activity, sodium malate was found to be important in both the inhibitory effect of *Angelicae Radix* and Juzen-taiho-to. The effects of the constituent crude drugs of Juzen-taiho-to other than *Angelicae Radix* on cisplatin toxicity were also investigated, and sodium malate was found to be the active constituent of *Ginseng Radix, Glycyrrhizae Radix, Astragali Radix, Atractylodis Rhizoma, Cnidii Rhizoma,* and *Paeoniae Radix.* However, some other active constituents also appeared to be present in *Cinnamomi Cortex* and *Hoelen.* In addition, the effect of sodium malate was markedly inhibited in *Rehmanniae Radix.* We prepared a Kampo formulation containing the crude drugs of Juzen-taiho-to other than *Rehmanniae Radix* and investigated the relationship between the sodium malate content and the inhibition of nephrotoxicity. The *Rehmanniae Radix*-free formulation was found to be more active than Juzen-taiho-to, suggesting that some constituents of *Rehmanniae Radix* suppressed the inhibitory effect of sodium malate on nephrotoxicity in the Juzen-taiho-to. Although the inhibitory constituent in *Rehmanniae Radix* remains to be identified, the role of sodium malate in the inhibition of nephrotoxicity by Juzen-taiho-to was clarified in the present study.

Key words cisplatin, sodium malate, nephrotoxicity, Juzen-taiho-to, *Rehmanniae Radix, Ginseng Radix,* Kampo medicine.

Abbreviations CDDP, cis-diamminedichloroplatinum II (cisplatin) ; BUN, blood urea nitrogen.

Introduction

In recent years, attention has been focused on therapeutic methods based on the concept of biochemical modulation. In the method, an anticancer agent is combined with other drugs in order to increase its antitumor effect and reduce adverse reactions by changing its pharmacokinetic profile.[1-5]

For the purpose of finding drugs to reduce adverse reactions to anticancer agents and improve the outcome of chemotherapy, we have already investigated the effects of Kampo formulations in mice given *cis*-diamminedichloroplatinum II (cisplatin, CDDP) and have shown that Juzen-taiho-to (十全大補湯) reduces adverse reactions, such as nephrotoxicity, bone marrow toxicity, gastrointestinal toxicity, and hepatotoxicity, without reducing the antitumor activ-

*〒422 静岡市谷田52-1
静岡県立大学薬学部漢方薬研究施設 杉山 清
52-1, Yada, Shizuoka-shi, Shizuoka 422, Japan

ity.[6] We have also investigated the effects of constituent crude drugs of Juzen-taiho-to and found that *Angelicae Radix* plays an important role in the effect of Juzen-taiho-to.[7] Furthermore, we isolated sodium malate from *Angelicae Radix* as an active constituent having inhibitory effect on the nephrotoxicity.[7] The ED_{50} of orally administered sodium malate for nephrotoxicity (determined using the blood urea nitrogen level) was 0.4 mg/kg, which was equivalent to about 65-fold the activity of *Angelicae Radix* (ED_{50} : 25.8 mg/kg). However, since the yield of sodium malate from our separation process was very low (0.0008 %), it could not be confirmed from the activity or yield that sodium malate is the active constituent of *Angelicae Radix.*[7]

In the present study, we investigated the content of sodium malate in Juzen-taiho-to and its constituent crude drugs and evaluated their effect on the CDDP nephrotoxicity for the purpose of clarifying the role of sodium malate.

Materials and Methods

Animals : Five-week-old male ddY mice (average weight : 25 g) were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and were kept in a room with a controlled temperature (23±0.5°C) and humidity (50±5 %) under a 12-h light/dark cycle. They were fed commercial mouse chow (MF : Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitume,* and were used after one week of acclimatization (average weight : 30 g).

Preparation of CDDP solution : CDDP was obtained from Sigma Chemical Co. (St.Louis, Mo, USA). The toxicity of CDDP depends on its aquation reactions. Greene *et al.*[8] found that the aquation reactions reached an equilibrium after 2 days at room temperature, and that the equilibrium was maintained for a long time in 0.9 % saline. Therefore, a solution of CDDP in sterile 0.9 % saline at a concentration of 0.5 mg/ml was prepared more than one week before injection.

Preparation of water extracts of Juzen-taiho-to and its constituent crude drugs : The constituent crude drugs of Juzen-taiho-to were purchased from Yamamoto Yakuhin Kogyo Co. Ltd. (Tokyo). A normal daily doses of Juzen-taiho-to and its constituent crude drugs were prepared as aqueous extracts by boiling the following crude drugs singly or together for 60 min : *Angelicae Radix* (3 g), *Hoelen* (3 g), *Glycyrrhizae Radix* (2g), *Ginseng Radix* (3 g), *Astragali Radix* (3 g), *Cinnamomi Cortex* (3 g), *Atractylodis Rhizoma* (3 g), *Paeoniae Radix* (3 g), *Cnidii Rhizoma* (3 g) and *Rehmanniae Radix* (3 g). After cooling, the extract was filtered and then lyophilized. The yield of each extract was as follows : Juzen-taiho-to (28.0 %), *Angelicae Radix* (33 %), *Hoelen* (1.2 %), *Glycyrrhizae Radix* (26.2 %), *Ginseng Radix* (36.8 %), *Astragali Radix* (25.6 %), *Cinnamomi Cortex* (5.5 %), *Atractylodis Rhizoma* (35.1 %), *Paeoniae Radix* (18.6 %), *Cnidii Rhizoma* (24.7 %) and *Rehmanniae Radix* (48.3 %). The yield of the extract of Juzen-taiho-to without *Rehmanniae Radix* was 19.9 %. The lyophilized materials were dissolved in water immediately before use.

Chemicals : Special-grade sodium malate and malic acid (Wako Pure Chemical Industries, Co., Tokyo) were used.

Treatment of animals : Animals were treated by a modification of the method reported previously.[6] Briefly, groups of 10 mice were inoculated with sarcoma 180 (S-180) cells (10^6/mouse) subcutaneously into the left thigh on day 1. Then CDDP (3.0 mg/kg/day) was given intraperitoneally to the mice on days 3,4,5,6,7,8,10,11, and 12. Sodium malate was given orally to the mice on days 3,4,5,6,7,8,10,11,12,13,14,and 15. Mice in the control group received water (p.o.) and 0.9 % saline (i.p.) instead. On day 17, the mice were anesthetized with ether and blood was collected from the inferior vena cava using a heparinized syringe. After centrifugation of the blood, the serum was analyzed to determine the blood urea nitrogen (BUN) level.

Sample preparation for HPLC : Water extracts of Juzen-taiho-to and each constituent crude drug (50 mg) were dissolved in water to make up a volume of 1.0 ml, and the resulting solution was filtered through a 0.45 μm disposable filter for HPLC (German Science Japan Co., Tokyo) and used as the sample. A volume of 10 μl of each sample was injected per HPLC run.

Determination of sodium malate by HPLC : The

HPLC apparatus was a CCPM (Toso Corporation, Tokyo) equipped with a controller (PX-8000), a thermostat (CO-8011), an ultraviolet detector (UV-8010), and a data processor (Chromatocoder 12, Toso Corporation). Sodium malate was determined by the method of Fujimura *et al.*[9] The operating conditions were as follows ; A TSK ODS80T column (4.6 mm i.d.×250 mm) (Toso Corporation) was placed in a column oven (40°C), and phosphate buffer (0.05M KH_2PO_4 : 0.05M H_3PO_4=1 : 1, pH3.0) was injected at a flow rate of 0.5 ml/min. The detection wavelength was 210 nm (UV). Since malates are all analyzed as malic acid by this method, the sodium malate content was calculated from a calibration curve which was previously constructed based on the assay values of malic acid.

Measurement of BUN: BUN was measured with a COBAS FARA (Baxter,Ltd.,Tokyo) by a spectrometric assay kid for urea nitrogen-HR-II (Wako Pure Chemical Industries, Ltd.).

Calculation of ED_{50} : The inhibition rate at each concentration was plotted on a logarithmic graph and the ED_{50} was calculated according to the least squares method.

Statistics : Student's *t*-test was used to evaluate the significance of differences between the experimental groups.

Results and Discussion

Sodium malate content of Juzen-taiho-to and its constituent crude drugs

Table I shows the malic acid content of Juzen-taiho-to and its constituent crude drugs determined by HPLC (Fig. 1). The usual daily dose of Juzen-taiho-to contained 79.5 mg (0.98 %) of sodium malate. In addition, the constituent crude drugs all contained sodium malate. The content was highest in *Angelicae Radix* (23.1 mg/usual daily dose), which had the gratest effect on nephrotoxicity, followed by *Ginseng Radix* (14.8 mg/usual daily dose), *Rehmanniae Radix* (10.7 mg/usual daily dose), and *Astragali Radix* (9.2 mg/usual daily dose) in that order. Furthermore, the total sodium malate content (80.5 mg) of the constituent crude drugs was found to be almost the same as that (79.5 mg) of Juzen-taiho-to. Although the efficiency of extraction of active constituents has been reported to change when various crude drugs are combined in Kampo formulations,[10,11] such a change was not noted in the present study.

Table I Content of sodium malate in the usual doses of Juzen-taiho-to and its constituent crude drugs.

	Crude drug (g)	Aqueous extract (g)	Content (mg)[a]
Juzen-taiho-to	29 g	8.12	79.5
Angelicae Radix	3	0.99	23.1
Ginseng Radix	3	1.10	14.8
Rehmanniae Radix	3	1.45	10.7
Cnidii Rhizoma	3	0.77	9.2
Glycyrrhizae Radix	2	0.52	6.0
Hoelen	3	0.04	6.0
Astragali Radix	3	0.74	4.8
Paeoniae Radix	3	0.56	4.5
Cinnamomi Cortex	3	0.17	1.1
Atractylodis Rhizoma	3	1.05	0.3

a) Content of sodium malate in the usual dose of aqueous extract.

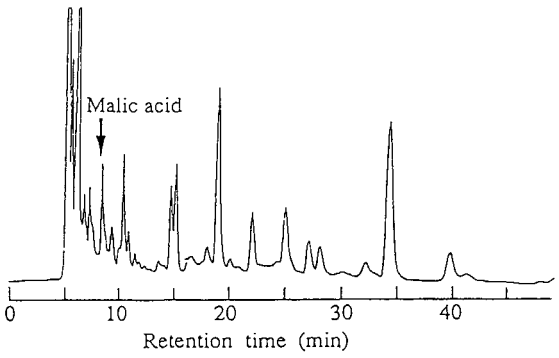
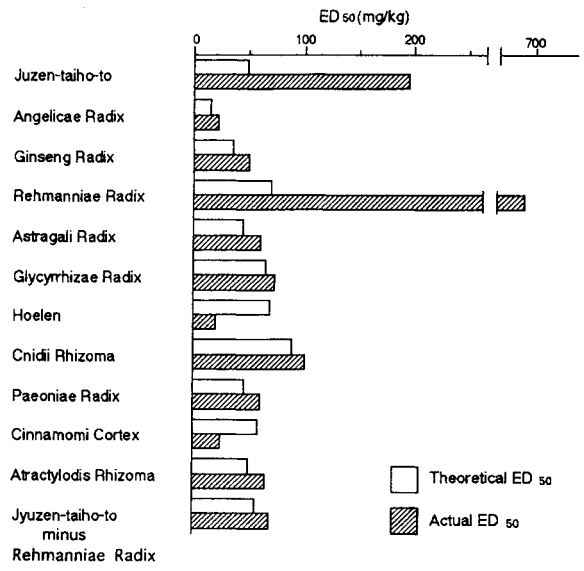


Fig. 1 HPLC of Juzen-taiho-to extract.
Conditions: Column ; TSK ODS-80T (4.6×250 mm), Column temp. ; 40°C, Eluent ; 0.05M KH_2PO_4/0.05M H_3PO_4 (1 : 1), Flow rate ; 0.5 ml/min, Detection ; UV-210 nm, Injection ; 10 μl (50 mg/ml)

Role of sodium malate in the inhibition of nephrotoxicity by Juzen-taiho-to and its constituent crude drugs

The ED_{50} of orally administered sodium malate and Juzen-taiho-to for the CDDP-induced nephrotoxicity was 0.4 and 200 mg/kg, respectively, while that of orally administered constituent crude drugs other



Fgi. 2 Sodium malate content and ED_{50} of Juzen-taiho-to and its constituent crude drugs.

than *Rehmanniae Radix* was lower than that of Juzen-taiho-to (see Actual ED_{50} in Fig. 2). In particular, *Angelicae Radix, Hoelen,* and *Cinnamomi Cortex* were more active and their ED_{50} values were 25.8, 13.5, and 19.6 mg/kg, respectively.

Fig. 2 also shows the theoretical ED_{50} of sodium malate calculated from the activity of each crude drug (ED_{50}) and the sodium malate content of each constituent crude drug. As shown in Fig. 2, the crude drugs could be classified into three groups. The measured activity was almost the same as the calculated value in the first group, including *Angelicae Radix, Ginseng Radix, Glycyrrhizae Radix,* and *Cnidii Rhizoma.* Since the activity corresponded to the sodium malate content, their active constituent was concluded to be sodium malate. In the second group, including *Hoelen* and *Cinnamomi Cortex,* the measured activity was higher than the calculated value. Such a finding suggested that some other constituent increasing the activity of sodium malate or having an inhibitory effect on the nephrotoxicity was present in the herbs of this group. In the third group, including Juzen-taiho-to and *Rehmanniae Radix*, the measured activity was lower than the calculated value. The activity of sodium malate was markedly depressed in Juzen-taiho-to, because the measured value was about one-fourth of the calculated one. This trend was even more obvious with *Rehmanniae Radix*, since the measured activity of sodium malate was only about one-tenth of the calculated value. Thus, some constituents of *Rehmanniae Radix* were considered to be related to the lower activity of sodium malate in Juzen-taiho-to.

We prepared a Kampo formulation containing 9 constituent crude drugs of Juzen-taiho-to excluding *Rehmanniae Radix* to investigate a role of *Rehmanniae Radix* in Juzen-taiho-to. As a result, 59.6 mg of sodium malate was found to be present in the usual daily dose of this formulation. This value was almost the same as that obtained by subtracting the sodium malate content of *Rehmanniae Radix* (10.7 mg) from that of Juzen-taiho-to (79.5 mg). As shown in Fig. 2, the measured ED_{50} of this formulation was 72 mg/kg, which was approximately the same as the theoretical value (58 mg/kg). Based on these results, *Rehmanniae Radix* was considered to be related to the inhibition of sodium malate in Juzen-taiho-to. It was suggested that sodium malate may be rapidly distributed in the blood and tissue after absorption into the body, bind to about 40 % of CDDP, from an intermediate diamminoplatinum (II) malate, and selectively reduce only the toxicity without affecting antitumor effect. And it was considered that such the selectivity of sodium malate for reduction of toxicity may be attributable to the difference in the distribution of sodium malate in the tissues. This suggests that the inhibitory substance in *Rehmanniae Radix* may inhibit the bind of CDDP and sodium malate or may change the distribution of sodium malate in the tissues. Studies are now underway to identify the inhibitory substance in *Rehmanniae Radix* and to elucidate the clinical significance of its inhibitory effect on sodium malate.

In the present study, the role of each constituent crude drug of Juzen-taiho-to was assessed using the effect on CDDP-induced nephrotoxicity as a yardstick. The activity of each crude drug (or the sodium malate in each crude drug) was unmodified in *Rehmanniae Radix*-free Juzen-taiho-to, while it was reduced by *Rehmanniae Radix* in standard Juzen-taiho-to. This new finding obtained by the present study seems to provide an insight into the complex effect of crude drugs in Kampo formulations.

和文抄録

本論文は，十全大補湯のシスプラチン毒性軽減効果における，活性成分リンゴ酸ナトリウムの役割を検討したものである。十全大補湯及びその配合生薬中のリンゴ酸ナトリウムの含量をHPLCで検討した。その結果，リンゴ酸ナトリウムは十全大補湯中に0.98％含有していること及びすべての配合生薬に含まれていること，ならびに配合生薬のうちでは当帰（23.1％）及び人参（14.8％）中に多く含有されていることがわかった。

次にシスプラチンの腎毒性に対するリンゴ酸ナトリウムの軽減効果を検討した結果そのED_{50}は0.4 mg/kgであり，十全大補湯及び最も強い活性を示した配合生薬当帰のそれぞれ約500倍及び65倍であることがわかった。これらの結果より，リンゴ酸ナトリウムは，十全大補湯及び当帰の腎毒性軽減効果における重要な役割を担っている成分であることが明らかになった。さらに，当帰以外の配合生薬におけるリンゴ酸ナトリウムの役割を検討した結果，人参，甘草，黄耆，川芎，芍薬，白朮においてリンゴ酸ナトリウムが活性本体であると考えられた。しかし，桂皮及び茯苓についてはリンゴ酸ナトリウム以外にも活性成分が存在する可能性が示唆された。また熟地黄においては，リンゴ酸ナトリウムの活性が何らかの成分により著しく抑制されていることが示唆された。そこで十全大補湯から熟地黄を除いた処方を調製し，活性を比較したところ，十全大補湯よりも強い抑制効果を示すことがわかった。このことより，十全大補湯においてもリンゴ酸ナトリウムの作用が，熟地黄により著しく抑制されていることが明らかになった。この熟地黄の成分については現在のところ不明であるが，本研究により，十全大補湯の腎毒性軽減効果において，リンゴ酸ナトリウムが重要な役割を演じていることが明らかになった。

References

1) Cadman, E.,Heimer, R., *et al.*: The Influence of Methotrexate Pretreatment on 5-Fluorouracil Matabolism in L1210 Cells. *J. Bio. Chem.* **256**(4), 1695-1704, 1981.
2) Atoh, M., Debra, M.Kloth, *et al.*: Modulation of Both Cisplatin Nephrotoxicity and Drug Resistance in Murine Bladder Tumor by Controlling Metallothionein Synthesis. *Cancer Res.* **53**(15), 1829-1832, 1993.
3) Sasaki, T.: Biochemical modulation. *Therapeutics* **72**, 1657-1664, 1990.
4) Morikage, T., Ohmori, T., *et al.*: Modulation of Cisplatin Sensitivity and Accumulation by Amphotericin B in Cisplatin-resistant Human Lung Cancer Cell Lines. *Cancer Res.* **53**, 3302-3307, 1993.
5) Chun-Ming Tsai, Shu-Huei Hsiao, *et al.*: Combination Cytotoxic Effects of cis-Diamminedichloroplatinum (II) and 5-Fluorouracil with and without Leucovorin against Human Non-Small Cell Lung Cancer. *Cancer Res.* **53**(15), 1079-1084, 1993.
6) Sugiyama, K., Ueda, H., Ichio, Y., Yokota, M.: Improvement of Cisplatin Toxicity and Lethality by Juzen-taiho-to in Mice. *Biol. Pharm. Bull.* **18**(1) 53-58, 1995.
7) Sugiyama, K., Ueda, H., Suhara, Y., Kajima, Y., Ichio, Y., Yokota, M.: Protective Effect of Sodium L-Malate, an Active Constituent Isolated from Angelicae Radix, on cis-Diamminedichloroplatinum (II)-Induced Toxic Side Effect. *Chem. Pharm. Bull.* **42**(12) 2565-2568, 1994.
8) Greene, R.F., Chatterji, D.C., Hiranaka, P.K., Gallelli, J.F.: Stability of cisplatin in aqueous solution. *Am. J. Hosp. Pharm.* **36**, 38-43, 1979.
9) Fujimura, K., Tsuchiya, M.,: Analysis of organic acids for discrimination of imported wines by ion chromatography. *BUNSEKI KAGAKU* **35**, 50 56, 1986.
10) Ohtsuka, Y., *et al.*: The Dictionary of Eastern Medicine (Toyo igaku-dai-jiten), Kodansya, Tokyo, pp.24-25, 1988.
11) Ohtsuka, K.: The Dictionary of Kampo (Kampo-dai-jiten), Kodansya, Tokyo, pp.6-7, 1975.