行政院國家科學委員會專題研究計畫　成果報告

開發以 Cisapride 為探針試藥來標定大白鼠體內細胞色素 3A 活性之研究:1.單點法之開發

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開發以 Cisapride 為探針試藥來標定大白鼠體內細胞色素 3A 活性之研究:1.單點法之開發

Investigation the potential roles of cisapride as a model substrate to assess CYP 3A activity in rat

計劃編號: NSC 93-2320-B-041-004

執行期間: 93 年 8 月 1 日 至 94 年 7 月 31 日

計劃主持人: 鄭靜玲　嘉南藥理藥理科技大學藥理學院藥學系

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一、中文摘要

近年來與 CYP3A 相關的藥物交互作用為國內外研究的熱門主題。此乃因為 CYP3A 為肝臟及小腸中含量最豐之細胞色素 P450，且有半數之藥品由此酵素代謝。

在臨床情況下，為瞭解體內代謝酵素活性，常利用藥物動力學的原理，選擇適當的藥品(即探針試藥)，並藉由統計學的方式，找出血中單點濃度中與清除率(Cl)或血漿濃度－時間關係圖之曲線下面積(AUC)最具關連的一點，當作臨床上的活性指標，進而達到臨床上需要快速瞭解病情並藉以調整劑量之依據。以細胞色素 3A 代謝酵素為例，現今最常被使用的探針試藥包括：抗生素類的紅黴素(erythromycin) 、鎮靜安眠的 midazolam 、及內生性的荷爾蒙代謝物 6β-hydroxy cortisol/cortisol 等。然而這些探針試藥或多或少都有其臨床上的限制：如需要使用放射性藥品的紅黴素、需使用臨床劑量的 midazolam 會造成病人的嗜睡狀態、而內生性的 6β-hydroxy cortisol/cortisol 也常有分析上干擾的問題發生，此數值一般認為僅代表部分肝臟 CYP3A 之活性。因此臨床上仍不斷有新的探針試藥的研究。最近文獻亦發現 Cisapride 口服劑量後之八小時或十二小時的單點濃度，也被報導可作為人體細胞色素 3A 單點探針(Lowry, 2003)。根據此想法，在藥物動力學臨床前試驗階段，為快速瞭解藥品體內藥物動力學特性，大鼠為最常使用的動物模式。在研究藥品代謝相關的交互作用時，亦喜使用此動物模式。出乎我們的意料之外，在過去的研究報告中將探針試藥的單點法使用在動物實驗的幾無報導。以我們過去研究的經驗，在研究此類細胞色素 3A 相關的藥品交互作用時，因其個體差異很大故所需之樣本數也須很大。又因大鼠之體型限制，採血量亦被限制。故若能找出探針試藥的單點法，則可有效減少生物檢體的數目，下降藥品血液濃度分析的難度，在訓練人員的困難度上將有所助益。並可加速研究結論之取得。

關鍵詞：平菩賜(Prepulsid®、Cisapride)、大鼠、單點法、藥物動力學、及藥品交互作用

二、Abstract

To evaluate a specific enzyme activity *in vivo*, it is quite common using an identified enzyme substrate as a probe in clinical situation. Most the time these probes have to fit the specified pharmacokinetics requirements. By means of an appropriate statistic correlation approach, the method can be further simplified by a single point concentration. Hence, the method can reduce sample number, save handling time, cost less, and even require less personnel and space.

Currently, the most popular *in vivo* CYP3A4 probes used in clinics are erythromycin, midazolam, and endogenous 6β-heydrosy cortisol/cortisol. However, none of them are idealized. For instance, carbon14 labeled erythromycin have to be

used, it may caused patients concern; therapeutic dose were required for midazolam, it may produce sedative and amnestic effects in some patients; endogenous 6β-hydroxy cortisol/cortisol may

only partially reflects liver CYP3A3/4 activity. Hence, one can see the new probe is proposed and studied. Recently, Lowry et al. (2003) demonstrated that cisapride also can be used as an potential *in vivo* probe in human. It was found that cisapride to norcisapride plasma concentration ratio at 8 hours and 12 hours were shown to accurately predict the area under the plasma concentration curve for cisapride.

In drug development process, rat was a very important animal model contributed to understand drugs biopharmaceutical characteristics - absorption, distribution, metabolism and elimination. It is also the species that we used to study drug-drug interactions. To our surprise, only very limited paper described about CYP3A single point method to probe CYP3A activity in rat. From our past experience, in studying CYP3A related drug-drug interaction, the number of rats must be big enough to compensate the large intersubject variation. In addition, limited by small size of rats, the plasma sample volume was been restricted. Hence a single point probe method could reduce the bioanalytical difficulty and might accelerate pace of this type studies. This year we propose that cisapride should be evaluated for its potential role as an *in vivo* model substrate to probe CYP 3A activity in rat.

MATERIALS AND METHODS

一、Materials

1、Purchased from BDH Laboratory Supplies，Poole，England

Acetonitrile (ACN，HPLC grade)

Methanol (MeOH，HPLC grade)

2、Purchased from J. T. Baker

Magnesium chloride4，6-hydrate，Crystal (MgCl2．6H2O, Lot N18H24)

Sodium phosphate，Monobasic，Monohydrate，Crystal (KOCO(CHOH)2 COONa．4H2O， Lot N03349)

3、Purchased from Riedel-deHaën，Germany

di-Sodium hydrogen phosphate-2-hydrate (Na2HPO4．2H2O，Lot 00770)

4、Purchased from Karayama Chemical，Japan

Acetic acid (CH3CooH，Lot A0945)

5、Sigma，St. Louis，MO，U.S.A.

β-Nicotinamide adenine dinucleotide phosphate，reduced form(β-NADPH，Lot 81K7059)

Urethane(Ethyl carbamate，Lot 51K 1269)

6、Purchased from Merck，Darmstadt，F.R. Germany

Sodium acetate (CH3COONa)

7、Purchased from Union Chemical Works LTD, Taiwan

Ethyl ether

8、Received from Lotus Medical Supply Inc, Taiwan as a gift

Cisapride

9、Purchased from Biomol Research Labs., Inc.. USA.

Delavirdine mesylate (Lot.P4251f)

二、Animal

Male Sprague-Dawley rats (250-350 g; obtained from the Animal Breeding Center of National Cheng Kung University) were maintained on standard laboratory pellets and

water *ad libitum*. The study protocol complied with the Institutional Guidelines on Animal Experimentation of National Cheng Kung University. It also complied with the institutional Guidelines on Animal Experimentation of Chia-Nan University of Pharmacy & Science.

三、Cisapride and norcisapride assay method development

Quantitaion of cisapride and its in vivo major metabolite – norcisapride in rat liver microsome and plasma were modified form our previous published method, using delavirdine as an internal standard (Cheng, Hu and Chou, 2002).

四、PK Studies of cisapride

To evaluate the cisapride/norcisapride blood levels, PK studies will be carried on SD rats. A femoral vein was cannulated (polyethylene tubing, PE-50) for blood sampling one day before PK study. On the day of PK studies, the designed dose 10 mg/kg of cisapride was given. Blood (about 200 μl) samples were collected at designed intervals from a femoral vein via the cannula The blood samples withdrawing time were before and 0, 0.17, 0.33, 0.67, 1, 1.5, 2, 3, 4, and 6 hours after administration. Blood samples were kept on a nitrogen tank until its levels being analyzed.

五、Data Analysis

The pharmacokinetic parameters of cisapride were calculated by a noncompartmental approach (Rowland and Tozer, 1995). Parameters will include, but are not limited to, area under the plasma concentration-time curve (AUC), clearance (CL), volume of distribution (Vd/f) , terminal phase half-life ($t_{1/2}$). CYP3A levels were measured by western blots. Single time point concentration of cisapride were correlated with AUC to determine to the best time describer.

六、Western Immunoblots of CYP3A

Hepatic and intestinal microsomes obtained from control and pretreated rats were solublized in sodium dodecyl sulfate (SDS), resolved by polyacrylamide gel electrophoresis according the method of Laemmli (1970), and then transferred to a nitrocellulose sheet. Western blot analysis using goat polyclonal (anati-CYP3A2 and anti-CYP2E1), antibodies that were purchased from Gentest Co. (MA, USA), and monoclonal antibody of Pgp, C219 and its control negative antibody were obtained from Centocor Diagnostics, Inc. (Malvern, PA). Immunoreactive protein bands were quantified by densitometry. (Cotreau *et al.,* 2000).

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RESULTS AND DISCUSSION

1. Cisapride and norcisapride assay method development

HPLC condition:

Mobile Phase (A) 50mM phosphate buffer (pH 4.0): acetonitrile = 35:65

Mobile Phase (A) 10mM phosphate buffer (pH 6.0): acetonitrile = 72:28

Flow rate: 1.0 ml/min with a mobile phase gradient program。

Fluorescence detection: Excitation 295 nm,

Emission 350 nm; Temperature: ambient;
Analytical column: C18
Detection limit of cisapride: 0.1 μg/mL

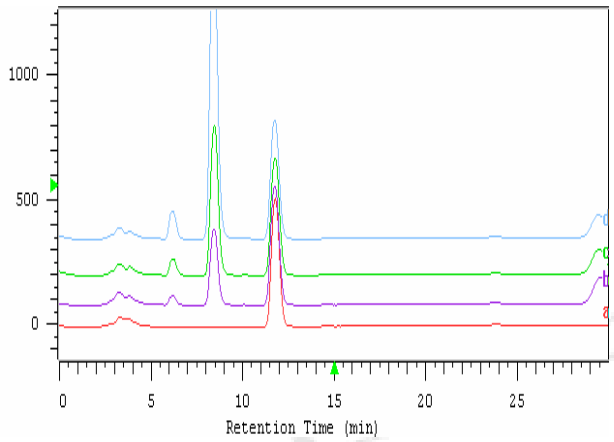

Fig.1 Chromatograms of cisapride and norcisapride before (a) and 15 minutes after incubation with cisapride at (b) 5 μM (c)10 μM and (d) 20 μM

2. PK studies

To investigated the CYP3A metabolic activity of cisapride in vivo, 10 mg/kg of cisapride was given via i.v. injection.. Induction CYP3A studies were fed with vitamin E TPGS consecutively 14 days before i.v. administered cisapride dose. The produced plasma concentration-time profiles were depicted in Fig.2. The resulted PK parameters were listed in Table 1.

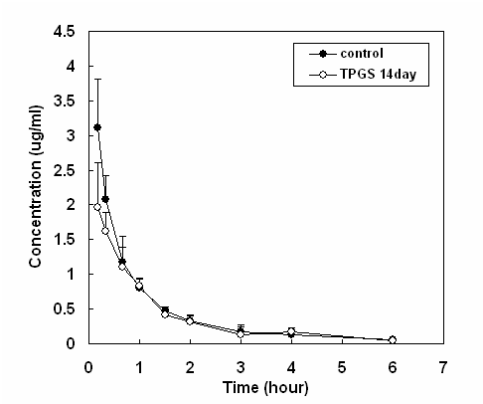

Fig. 2.Cisapride plasma concentration-time proliles after i.v. dosing 10 mg/kg.

Table 1. Listed PK parameters of Cisapride after i.v. dosing 10 mg/kg

	N	Rat (g)	C_{max} (μg/mL)	$T_{1/2}$ (hr)	AUC (hr*μg/mL)	CL (mL/min/kg)
Control	3	292 ± 5	4.91 ± 2.24	1.47 ± 1.03	3.13 ± 0.41	53.92 ± 7.31
TPGS 14d	4	292 ± 4	2.48 ± 1.17	0.76 ± 0.38	2.34 ± 0.67	76.14 ± 23.09

3. Data Analysis

The relationship between AUC and Cisapride Concentration were evaluated by correlation. As depicted in Fig. 3, it was found that the cisapride concentration on 0.33h is well correlated with AUC, r = 0.9261.

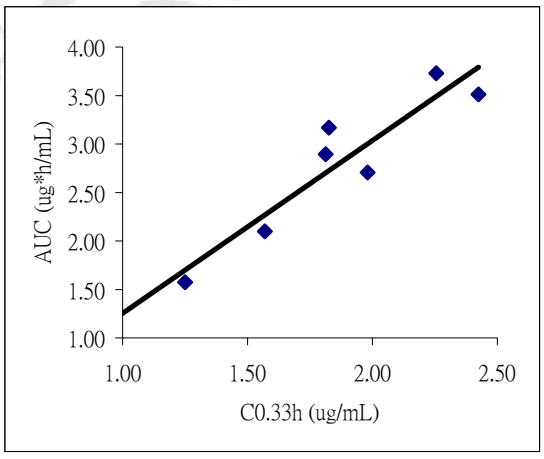

Fig. 3. Correlation between cisapride concentration at 0.33h and AUC.

4. Western Immunoblots of CYP3A

The results of western immunoblots of

CYP3A of control group and induced with TPGS group were shown in Fig.4. Based on the results, after fed with TPGS 14 days, rat CYP3A2 protein were about 2 fold increased .

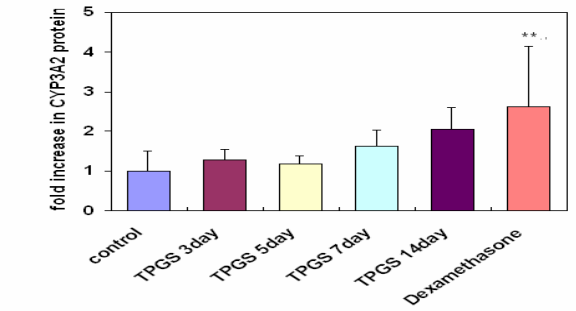

Fig.4. Hepatic CYP3A2 protein levels after oral dosed vitamin E TPGS.

CONCLUSION

Based upon above results，a valid and sensitive HPLC method was developed and could be utilized in quantification of cisapride and norcisapride concentration in both microsome system and also in rat plasma. As shown in our pharmacokinetic studies, even in the low dose as 10 mg/kg, this developed HPLC method could detected the whole plasma concentration - time profile. Using a correlation approach, it was found the cisapride plasma concentration at time 0.33 hr correlated well with AUCinf. It is possible to use this specific time cisapride plasma concentration as an rat CYP3A2 indicator. Our preliminary studies in western immunoblots of CYP3A2 also shown the tested animals were with scattered CYP3A2 activity. However, to evaluate the performance of this single time point approach, further studies are required.

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