行政院國家科學委員會專題研究計畫　成果報告

從生物藥劑學觀點研究維生素 E 對細胞色素相關之藥品交互作用

計畫類別：個別型計畫
計畫編號：NSC91-2320-B-041-014-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：嘉南藥理科技大學藥學系

計畫主持人：鄭靜玲

計畫參與人員：周辰熹、何孟澔

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行政院國家科學委員會補助專題研究計畫成果報告

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※ 從生物藥劑學觀點研究維生素 E 對細胞色素相關之藥品交互作用 ※

※ Biopharmaceutical Considerations on CYP3A Related ※

※ Drug-Drug Interactions: Effects of Vitamin E TPGS ※

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計畫類別：☒ 個別型計畫 □整合型計畫

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計畫主持人：鄭靜玲

共同主持人：

計畫參與人員：周辰熹、何孟潺

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□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：嘉南藥理科技大學藥學系

中 華 民 國 九十二 年 十 月 二十五 日

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從生物藥劑學觀點研究維生素 E 對細胞色素相關之藥品交互作用

Biopharmaceutical Considerations on CYP3A Related Drug-Drug Interactions: Effects of Vitamin E TPGS

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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。然而不像其他的細胞色素 450 iso-enzymes，已從藥物遺傳學(pharmaco-genetics)的研究中，找出許多與藥品活性有直接相關的不同對偶基因，因而可辨認出高危險性的藥品使用患者。相反的，至今雖有許多致力於 CYP3A 基因多型性的研究，然這些研究結果顯示發現的新基因型不能與 CYP3A 的活性直接相關，仍不能解釋為何 CYP3A 的活性具相當大的個體差異。重新檢視細胞色素對藥物的催化步驟，並將 CYP3A 與其他的細胞色素 450 isoenzymes 做比較後發現：CYP3A 為肝臟及小腸中含量最高之代謝酵素。此點是 CYP3A 被強調其在藥物代謝扮演一重要角色之因。

過去多認為口服劑型中之賦型劑不被吸收，故不會影響藥品的吸收速率。但新的製藥技術與藥品傳輸理論，使賦型劑與藥品間的關係趨向複雜，也使賦型劑對主成分藥劑的影響研究日趨重要。而其中曾有文獻報告維他命 E 的新型人工化學成份 TPGS 可能對 CYP3A 的代謝會有抑制的影響。TPGS 近來大量用於難溶性藥品製劑並兼具臨床療效，於製劑方面，被用為抗氧化劑、助溶劑、乳化劑、及吸收促進劑。這是利用其具有親水性及親油性基團，似界面活性劑之作用來增進藥品的吸收。於臨床使用方面，除可作為水溶性的維生素 E 來源外，對其降膽固醇，抗癌活性，抗氧化性及預防血小板凝集功能的研究更是日益增加。。而近來對油性製劑的重視，使本對其他藥品吸收的影響評估日趨重要。

由於先前的文獻顯示本賦之代謝可能與 CYP3A 有關。根據過去的經驗，此類藥劑長期使用，可能會誘發體內 CYP3A 的量。故在本研究中，我們欲了解長期使用 TPGS 對 CYP3A 肝臟含量與微粒體體外活性的影響。

我們在實驗組中連續餵食大白鼠 TPGS 分別 7 與 14 天，進行不同天數的 CYP3A 誘導反應，並與基礎對照組餵食給藥溶劑(生理食鹽水)與正相對照組(餵食 dexamethasone 四天)比對，藉由西方點墨法觀察相對應肝中細胞色素蛋白質含量的變化，並藉由體外微粒體速率試驗評估 CYP3A 體外活性變化，

由兩對照組的實驗結果顯示，本實驗室已確立 CYP3A 蛋白含量誘發與否與體外活性變化的評估指標。由西方點墨法之實驗結果可見明顯蛋白質含量的增加，而體外活性數據也證明實驗組的 V_{max} 比控制組大且兩祖的 k_m 並無明顯變化。由上述結果可知:長期餵食維生素 E TPGS 非常可能誘發 CYP3A 代謝酵素的表現，其體內影響正進一步評估中。

關鍵詞：CYP3A，維他命 E TPGS，藥物交互作用。

二、Abstract

Many synthetic or natural compounds can improve the intestinal absorption of hydrophilic and/or lipophilic drugs. Among these compounds, bile salts, fatty acids, and surfactants have been verified as potent absorption enhancers (Sakai, 1997). Vitamin E TPGS, D-α-tocopheryl polyethylene glycol 1000 succinate, is a derivative of vitamin E. Consisting of a hydrophilic polar group (tocopherol succinate) and a lipophilic alkyl group (polyethylene glycol), vitamin E TPGS is structurally similar to a conventional surface-active agent. With this chemical nature, TPGS has been introduced its pharmaceutical use as an antioxidant, a solubilizer, an emulsifier, an absorption enhancer, and as a water-soluble source of vitamin E. Additionally, in recent years there is a growing clinical interest in vitamin E for their cholesterol lowering effect, anticancer activities, antioxidant properties and anti-aggregation of blood platelets. Recently, Parker *et al*. (2000) reported a CYP3A-dependent mechanism of tocopherols to water-soluble carboxychromans, which can be then excreted in urine. The same paper also demonstrated sesamin inhibit γ–tocopherol to γ–carboxychromans by CYP3A *in vitro*, which explain the elevated concentrations of tocopherols in plasma and tissue when vitamin E was coadministered with sesame seed/oil to rats. Results from this study indicated the possibility of Vit. E may play a competitive substrate role on other CYP3A metabolized drugs. It also leads the speculation that long term use of vitamin E TPGS may result increase in CYP3A level. For this purpose testosterone, a typical CYP3A substrate, will be selected as an in vitro model drug to evaluate the potential induction effect of vitamin E TPGS on CYP3A activity. The protein content was evaluated by western blot method using a specific monoclonal antibody.

Our results demonstrate that the established method can successfully distinguish the control group and the positive control group (dexamethasone) in protein levels and in enzyme activity experiment (elevated V_{max} but not k_m) using testosterone as in vitro probe. As we compared the results from tested group and control group, similar pattern to positive control group results were also obtained. It is concluded that long-term use vitamin E TPGS can induce CYP3A level. This phenomenon is possible caused by homeostasis control. Since vitamin E supplement is very popular in Taiwan, the resulted CYP3A-related drug interactions should pay more attention. The potential drug-drug interaction is currently under investigated.

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 ($MgCl_2$. $6H_2O$, Lot N18H24)

Sodium phosphate, Monobasic, Monohydrate, Crystal ($KOCO(CHOH)_2COONa$. $4H_2O$, Lot N03349)

3、Purchased from Riedel-deHaen, Germany

di-Sodium hydrogen phosphate-2-hydrate (Na_2HPO_4. $2H_2O$, Lot 00770)

4、Purchasrf 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、Purchasrf from Merck, Darmstadt, F.R. Germany

Sodium acetate (CH3COONa)

7、Purchasrf from Union Chemical Works LTD, Taiwan

Ethyl ether

二、Animal treatment

Experiments were performed on SD male rats, 250-350 g. All rats were divided into 4 groups: (1) control – orally administered normal saline only; (2) positive control – orally administered dexamethasone for 4 days; (3) 7 days group – orally administered for 7 days normal saline and 7 days vitamin E TPGS; (4) 14 days group – orally administered for 14 consecutive days vitamin E TPGS. At the 15th day, all rats will be sacrificed humanly and following the procedure of microsome preparation.

三、Method for preparation of microsome

本研究目前利用 Male Sprague-Dawley rats(購自成大醫學院動物中心)的肝臟進行 microsomes 製備。其使用試劑為：Tris(hydroxymethyl) methylamine(BDH Laboratory Supplies)、Sodium acetate (Merck，Germany)、Sucrose (Merck，Germany)和 Glycerol (關東化學株式會社, Japan)。使用灌流手術方式先將肝臟血液趕出，獲得的肝臟將其剪碎並使用組織均質機(Glas-ColR Terre Haute，U.S.A.)均質之，藉由蔗糖梯度法使用高速離心機(RC-5C，Sorvall® Instruments)及超高速離心機(L7-65 Ultracentrifuge，Beckman coulter™)將 microsomes 分離。

四、In vitro protein level and enzyme activity evaluation：

1. Lowry Method for normalization of total protein level

使用的試劑包括：Sodium hydroxide (Mallinckrodt，Baker)、Folin-ciocalteu's phenol reagent(Fluka)、Kupfer(II)-sulfat-5-hydrate(Riedel-deHaen，Germany)及 Sodium carbonate anhydrous(Riedel-deHaen，Germany)，其 preotein 的定量是使用 Bovine serum albumin (BSA，Sigma)作為標準品，測量在 760 nm 之 UV 析光

2. Evaluation of CYP3A in vitro activity by testosterone & its metabolite 6β-OH testosteron

用 CYP3A 的典型受質 testosteron 進行 *in vitro* incubation 試驗，且藉由 HPLC 的分析觀察肝臟代謝活性之變化。其 HPLC 系統包括：自動取樣器(HITACHI L-7200 Autosampler)、幫浦(HITACHI L-7100 Pump)、界面控制器(HITACHI D-7000 Interface)、偵測器(HITACHI L-7420 UV Detector)、管柱(分析：Luna 5μ C18 Column 4.6×250mm (phenomenex®))和 保護：ODS Guard column (H5ODS-10C，Hichrom Ltd))、積分系統(D-7000 HPLC System Manager (HSM))。 藉由不同總蛋白質含量(25, 50 and 100 μg)、不同藥品濃度的投與(5-500 μM)和不同 incubation time:0.5, 1 ,3 ,5, 10, 15, 30 min 觀察肝臟酵素代謝情形。來確定最後的最適化條件。

3. Evaluation of CYP3A protein level by western blot

Hepatic microsomes obtained from control and pretreated rats will be 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 will be purchased from Gentest Co. (MA, USA). Immunoreactive protein bands will be quantified by densitometry. (Cotreau *et al.*, 2000).

4. Vitamin E Assay Method Development

Determination of vitamin E plasma/urine levels were modified from method of Yap (1999), or Koo and Noh (2001).

RESULTS AND DISCUSSION

1. Lowry Method:

To measure the total protein in different batches microsome as a normalized basis, lowry method was employed. The standard curve was dipicted here in Fig. 1. Linear concentration range of total protein was between 0.01-0.25mg/ml.

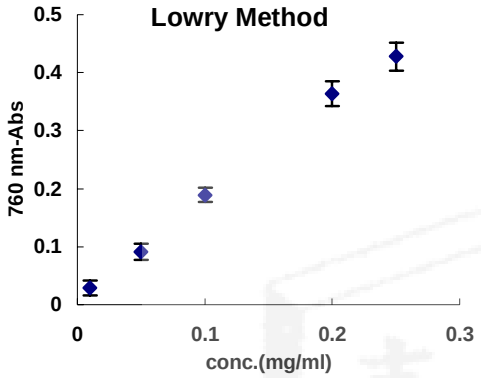

Fig. 1.: Lowry method:

R^2:0.997, intercept:0.013, slpoe:1.699

2. 評估CYP3A 體外活性的testosterone 微粒體培養法

To quantification the metabolizeing activity of CYP3A from microsome, we first develop a specific assay method for testosterone and its specific metabolite, 6β-OH testosterone, using delavirdine as an internal standard. The resulted carlibration curve was shown in Fig. 2.

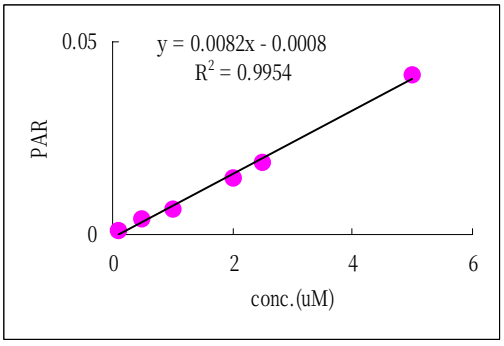

Fig. 2. Calibration curve of 6β-OH testosterone in rat microsome.

To investigated the CYP3A metabolic activity of testosterone, different concentrations of testosterone 5-100 μM will be incubated under specific microsome conditions (total protein 25, 50 and 100 μg). The appropriate total protein amount and incubation time were determined from Figs 3 to be 50 μg and 10 minutes, repectively.

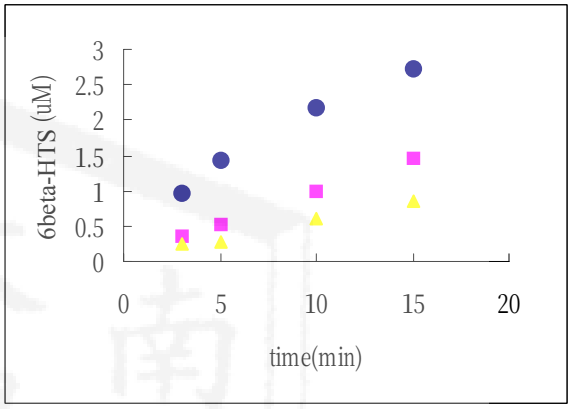

Fig. 3.Formation of 6β-OH testosterone vs time at testosterone 100 uM and 25, 50, and 100 μg total protein

Based on above observation, total protein content 50 ug , testosterone concentration were 5-100 μM and incubation time for 10 minutes were selected as the final assay condition.

In vitro activity assay for all groups were depicted in Fig.4.

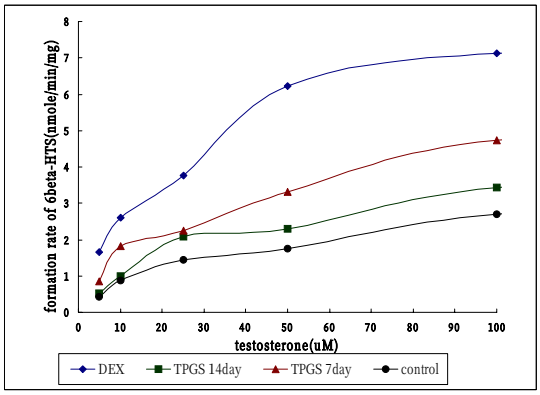

Fig.4 Formation rate of 6β-OH testosterone vs.

different incubation concentration of testosterone.

Data were then transformed to determine the V_{max} and k_m ， the resulted figure and table were shown in Fig. 5 and Table 1.

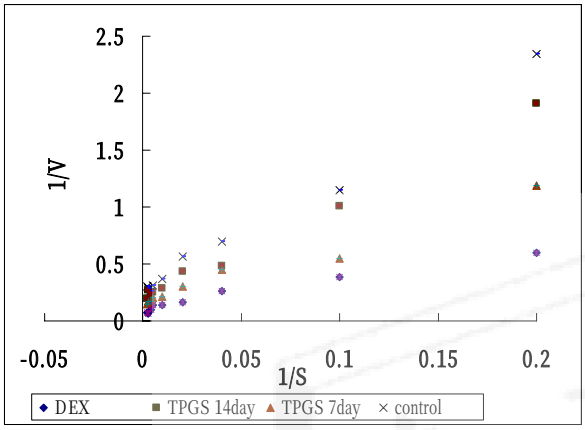

Fig. 5. Using double reciprocal method to determine V_{max} and k_M of the microsomal systems.

Table 1.Comparison of maximum velocity and Mechaelis-Menton Constant in different groups

	Control	DEX	TPGS 14d	TPGS 7d
slope	10.12	2.60	8.38	4.92
intercept	0.27	0.10	0.21	0.17
V_{max}	3.74	9.64	4.85	5.96
k_M	37.88	25.06	40.62	29.32

3. 用西方點墨法評估 CYP3A 微粒體 CYP3A 蛋白質含量

不同分組的試驗動物經不同方式的給藥處理後，於第 15 天一起犧牲，所得之微粒體於校正總蛋白含量後，進行西方點墨法藉以了解活性增加的原因。所得結果顯示於 Fig. 6.。與對照組比對，投與典型誘導劑 DEX4 天後，可見蛋白質含量明顯增加。相同的在連續投與 TPGS 7 及 14 天後，蛋白質含量也比對照組明顯增加。但二組中又以餵食 7 天組之效果較明顯。

Fig. 6. 西方典墨法顯示不同給藥組的蛋白質含量的變化。

4. Vitamin E Assay Method Development

HPLC condition:

mobile phase: MeOH-MQ=99:1

flow rate: 1.5ml/min

UV detection: 290nm

temperature: 室溫

column: phenomex C18 (250*4.6mm)

Drug: alpha, delta, gama-tocopherol溶於ethanol中分析

配成10uM,打10ul分析

result:

retention time:delta-tocopherol 8.98min

gama-tocopherol 10.50min

alpha-tocopherol 12.10min

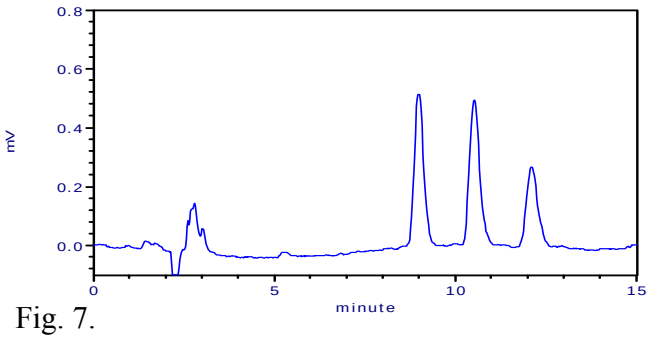

Fig. 7. Choromatograph of δ–,γ–, α–tocopherols.

CONCLUSION

由上述結果顯示，本實驗室已初步完成 testosterone 在微粒體系統的定量分析方法，並建立以其評估肝及腸微粒體中 CYP3A 體外代謝活性的系統。預試驗結果顯示長期投與 TPGS 的情況下，在給予劑量 7 天及 14 天後，都觀察到 CYP3A 的活性增加。顯示有酵素誘發反應。利用 testosterone 為探針藥品進行體外微粒體活性評估也發現 V_{max} 確有增加，k_M 的變化則較不明顯。本實驗結果顯示長期服用維生素 E TPGS 對 CYP3A 代謝效素確有誘導之效果，而長期投與後對其他經 CYP3A 代謝藥品的影響仍待進一步的評估。而本實驗室現正發展定量 tocopherols 及 TPGS 的血中濃度分析方法中，初步結果顯示於 Fig. 7。

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