

P-2020/44705

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIRECCIÓN DE NUEVAS CREACIONES

E. S. D.

Re: Expediente No. **NC2020/0011188**
Solicitud de patente a nombre de **SINEW PHARMA INC.**

Yo, CAROLINA DAZA MONTALVO identificada como aparece al pie de mi firma, domiciliada en la Carrera 11 No. 86-53, obrando como apoderada de la sociedad **SINEW PHARMA INC**, domiciliada en Taiwán, China, solicitante de la patente de la referencia, en respuesta al Oficio No. **12367** notificado mediante notificación electrónica el 17 de julio de 2024, relacionado con el tercer concepto técnico de fondo, atentamente me permito presentar la siguiente información:

1. Título

En primera medida, con respecto a la observación sobre la falta de relación del título con el objeto de la solicitud contenido en la descripción y las reivindicaciones, me permito señalar que, siguiendo la sugerencia del Examinador, el solicitante ha modificado el título como se muestra a continuación:

“COMPOSICIONES QUE COMPRENDEN NARINGENINA, KAEMPFEROL O LUTEOLINA
PARA REDUCIR LA TOXICIDAD DEL ACETAMINOFÉN (APAP)”

Teniendo en cuenta que el nuevo título refleja de una mejor manera el objeto de la solicitud, amablemente solicito que la objeción al respecto se dé por superada.

2. Modificación a la solicitud

Con fundamento en la facultad que confiere el Artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial un nuevo capítulo reivindicatorio, el cual consta de

cinco (5) reivindicaciones, el cual reemplaza completamente la versión previamente presentada.

Las modificaciones se resumen a continuación:

- 2.1** Se ha incluido la nueva reivindicación 4 dirigida a una composición de las reivindicaciones precedentes que adicionalmente comprende una cantidad terapéuticamente eficaz de APAP. Esta nueva reivindicación encuentra soporte en la página 5, líneas 23 y 24 y página 8, líneas 27 a 30 de la descripción.
- 2.2** Se ha incluido la nueva reivindicación 5 dirigida a una composición de las reivindicaciones precedentes en la que el ingrediente activo está incluido en una cantidad de 1 a 200 mg. Esta nueva reivindicación encuentra soporte en la sección 1.2. de la página 9 de la descripción.

El solicitante se permite manifestar que las modificaciones presentadas en esta oportunidad no implican una ampliación de la protección que correspondería a la divulgación contenida en la solicitud inicial, por lo que es posible afirmar que el capítulo reivindicatorio presentado junto a este memorial se ajusta a las disposiciones del Artículo 34 de la Decisión 486/00.

3. Pago de tasas

Considerando que el presente memorial se presenta junto con modificaciones a las reivindicaciones, se anexa el comprobante de pago correspondiente a la tasa de modificación, según lo establecido en la Resolución No. 59669 de 2020.

Adicionalmente, me permito resaltar que junto a la presente respuesta no se realiza el pago de la tasa por concepto de reivindicaciones adicionales, toda vez que el nuevo capítulo reivindicatorio tiene menos de 10 cláusulas.

4. Claridad, concisión y soporte de nuevas reivindicaciones

El solicitante manifiesta que mediante la presentación del nuevo capítulo reivindicatorio quedan subsanadas la totalidad de las objeciones, de modo tal que las nuevas reivindicaciones proporcionan claridad y concisión a la invención descrita, a la vez que cuentan con el debido sustento en la descripción de la solicitud.

Con respecto a las objeciones puntuales del examinador se presentan las siguientes

aclaraciones:

- 4.1** El Examinador considera que las reivindicaciones 1 a 3 no se encuentran sustentadas en la descripción, dado que la solicitud solo demuestra que se administró una serie de compuestos a *"(...) una dosis de menos de o equivalente a 200 mg por 60 kg del peso corporal de la persona de la administración de dosis oral individual (...)"* lo cual se relaciona con las cantidades en las que se administran los compuestos, más no con que se desarrollen composiciones farmacéuticas.

El Solicitante manifiesta su desacuerdo. La descripción describe cada componente, sus efectos, así como una composición que los contiene, por ejemplo, en las páginas 4, líneas 37 y 38, página 5, líneas 1 a 3, página 6, líneas 14 a 16, Tabla 1, página 11, líneas 36 a 38 y página 12, líneas 1 a 6 de la descripción.

La descripción también describe en las páginas 7 y 8 que, para el propósito de transporte y absorción, una cantidad efectiva de un ingrediente activo de acuerdo con la presente invención puede formularse con un vehículo farmacéuticamente aceptable para formar una forma adecuada de una composición farmacéutica y proporciona ejemplos de la forma de una composición farmacéutica tal como tabletas, píldoras, polvo, pastillas, paquetes, trociscos, elixires, suspensiones, lociones, soluciones, jarabes, cápsulas de gelatina suave y dura, supositorios, fluido esterilizado para inyección y polvo empaquetado.

La descripción enseña además en la página 8 que la preparación de una composición parenteral apropiada bajo condiciones estériles puede lograrse con técnicas farmacológicas estándar bien conocidas por las personas expertas en la materia.

La persona normalmente versada en la materia puede comprender la presente invención y preparar la composición como se reivindica basándose en la divulgación de la descripción en combinación con su conocimiento.

En consecuencia, se solicita dar por superadas las objeciones en este aspecto.

- 4.2** El Examinador asegura que el capítulo reivindicatorio no es claro ni conciso puesto que no se menciona la concentración o cantidad de cada uno de los compuestos en la formulación, abarcando así un indeterminado número de composiciones que no se encuentran debidamente sustentadas en la solicitud.

El Solicitante manifiesta su desacuerdo. La novedad y nivel inventivo de la composición reclamada reside en los ingredientes que se combinan y no en las proporciones específicas entre ellos. En este sentido, la reivindicación independiente 1 se refiere a un grupo limitado de ingredientes adecuadamente definidos por su nombre.

Adicionalmente, la cantidad de los ingredientes puede cambiar dependiendo de diversos motivos, tales como la vía y frecuencia de administración, el peso corporal y el individuo que recibe dicho producto farmacéutico y el propósito de la administración. La persona versada en la materia podrá determinar la cantidad en cada caso en función de la divulgación de la descripción, los métodos establecidos y su propia experiencia.

Ahora, la descripción menciona en la sección 1.2. de la página 9 que la cantidad de ingrediente activo puede ser menor o equivalente a 200 mg por persona de 60 kg de peso corporal. En consecuencia, se ha incluido la nueva reivindicación dependiente 5 dirigida a esta modalidad de la invención.

Teniendo en cuenta que el capítulo reivindicatorio es claro y conciso, se solicita dar por superadas las objeciones en este aspecto.

5. Descripción

El examinador indica que la descripción no divulga la materia reclamada de manera suficiente para que una persona normalmente versada en la materia pueda ejecutarla o reproducirla, por lo que no cumpliría con las disposiciones del Art. 28 de la Decisión Andina 486.

En particular, el Examinador considera que la solicitud no demuestra el desarrollo de ninguna composición farmacéutica, por el contrario, solo evidencia ensayos biológicos en los que se administra una serie de compuestos a una dosis de menos de o equivalente a 200 mg por 60 kg del peso corporal, lo que no permite que una persona versada en la materia pueda desarrollar composiciones farmacéuticas como lo pretende el solicitante.

El Solicitante manifiesta su desacuerdo y reitera que la descripción divulga las características de la presente invención de manera suficiente y proporciona ejemplos representativos para respaldar la viabilidad de la invención reivindicada.

La invención puede ser entendida y llevada a cabo por una persona normalmente experta en la técnica basándose en la divulgación de la descripción (*páginas 4, líneas 37 y 38, página 5, líneas 1 a 3, página 6, líneas 14 a 16, Tabla 1, página 11, líneas 36 a 38 y página 12, líneas 1 a 6*) junto con el conocimiento y la tecnología convencionales en el estado de la técnica.

Por lo tanto, la descripción proporciona toda la información necesaria para llevar a cabo la invención y cumple con los requisitos legales establecidos en la Decisión Andina 486.

6. Nivel inventivo

El examinador establece en su concepto técnico que las antiguas reivindicaciones 1 a 3 no cumplirían con el requisito de nivel inventivo a la luz de las enseñanzas de los documentos EP3069733 (**D1**) y US20140038921 (**D4**).

El Examinador considera que la diferencia entre la invención de la reivindicación 1 y la composición de D1 es la combinación con otros agentes que reducen la hepatotoxicidad generada por el metabolismo del CYP2E1 en el hígado, como la naringenina, el kaempferol o la luteolina.

Sin embargo, el Examinador considera que dicha diferencia no proporciona un efecto técnico diferente al que ya proporcionaban las composiciones D1, que ya reducían la hepatotoxicidad del paracetamol con disminuciones estadísticamente significativas en los niveles de AST, ALT, GSP. Además, el examinador reitera que la solicitud no proporciona evidencia del desarrollo de una composición farmacéutica que comprenda las combinaciones reivindicadas.

Finalmente, el examinador afirma que dicha diferencia ya se anticipa en D4, que demuestra la toxicidad reducida de un fármaco antituberculoso cuando se combina con un inhibidor de CYP2E1 (Kaempferol), manitol, sacarina, sucralosa, fosfato dicálcico o crospovidona.

Con respecto a lo anterior, me permito resaltar las disposiciones del artículo 18 de la Decisión 486 de la Comisión de la Comunidad Andina, que establecen que “*se considerará que la invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica*”.

Lo anterior significa que el análisis de nivel inventivo **NO** es una comparación de los objetos de las anterioridades y la solicitud en estudio. Por el contrario, el análisis de nivel inventivo **debe demostrar** la obviedad de la invención y establecer cómo ésta no contiene un salto cualitativo, que demuestre su altura inventiva, teniendo en cuenta que se hace **necesario demostrar** que la invención analizada carece de nivel inventivo y hubiese sido obvia para una persona versada en la materia.

Ahora bien, dichas condiciones no se cumplen para el caso en cuestión, pues, tal como se mostrará más adelante, las características técnicas mencionadas en las nuevas reivindicaciones no pueden derivarse de manera obvia o evidente a partir de las enseñanzas del estado de la técnica representadas en los documentos D1 y D4, y, por lo tanto, la materia reclamada es inventiva a la luz del arte previo.

Como reconoce el examinador, D1 no revela composiciones con naringenina, kaempferol ni luteolina. Sin embargo, el examinador afirma que las personas expertas en la materia estarían motivadas a incluir naringenina, kaempferol y luteolina de D4 en la composición de D1, porque D4 enseña que estos compuestos son inhibidores de CYP2E1.

El Solicitante manifiesta su desacuerdo. En primer lugar, no cualquier compuesto inhibidor de CYP2E1 funciona como agente para reducir la toxicidad causada por APAP. Como se muestra en D1, Tablas 2-3, el Tween 20 exhibe una alta actividad inhibidora de CYP2E1 ($80,6 \pm 1,9$ y $55,4 \pm 1,9$, $16,5 \mu\text{M}$), pero no muestra un efecto protector en la reducción de la hepatotoxicidad de APAP como evidencia Kelava et al. (*Canadian journal of physiology and pharmacology*, 88(10), 960-967, Anexo 1).

Kelava et al. describe los efectos de varios vehículos farmacológicos sobre la lesión hepática inducida por paracetamol. En particular, la Tabla 1 muestra que el nivel de ALT en el grupo de solución salina sin administración de paracetamol es de $20 \pm 1,5$ U/L y que en el grupo de solución salina con administración de paracetamol se eleva a $6747,5 \pm 2290,4$ U/L, lo que indica que se ha producido daño hepático por paracetamol; mientras que en el grupo de Tween 20, el nivel de ALT sigue siendo tan alto como $5268,6 \pm 2512,6$, lo que indica que el Tween 20 no reduce la hepatotoxicidad causada por paracetamol.

Como evidencia de lo anterior, también se hace referencia al resumen de Kelava et al., línea 12, y la página 3, Resultados, líneas 8-9. En concreto, aunque el Tween 20 es un

potente inhibidor del CYP2E1, no puede reducir la hepatotoxicidad causada por el acetaminofeno como se esperaba.

Ahora, el documento D4 describe una composición farmacéutica para tratar enfermedades tuberculosas que comprende uno o más fármacos antituberculosos, como isoniazida, rifampicina, pirazinamida y etambutol, y cantidades farmacéuticamente eficaces de sustancias que pueden reducir los efectos secundarios de los fármacos antituberculosos.

Sin embargo, el documento D4 no se refiere a una composición para reducir la toxicidad causada por APAP. De hecho, los fármacos antituberculosos y la APAP no son lo mismo. Distintos fármacos implican distintos mecanismos para provocar efectos secundarios. Algunos ingredientes útiles para reducir la toxicidad causada por los fármacos antituberculosos pueden no ser útiles para reducir la toxicidad causada por APAP; algunos incluso aumentan el riesgo de toxicidad.

Por ejemplo, el documento D4 describe que la quercetina, como inhibidor del CYP2E1 o de la amidasa, es útil para reducir los efectos secundarios de los fármacos antituberculosos. Sin embargo, Pingili et al. (*Drug Development and Industrial Pharmacy*, 41(11), 1793-1800, Anexo 2) describe que la quercetina, puede aumentar la exposición al paracetamol (acetaminofén) y aumentar el riesgo de toxicidad hepática (título, resumen, página 7, columna izquierda, líneas 16-18).

En estas circunstancias, la persona versada en la materia no estaría motivada a combinar D1 con D4 para incluir los ingredientes para reducir la toxicidad causada por los fármacos antituberculosos en la composición de D1 para reducir la toxicidad causada por APAP y llegar a la presente invención.

En consecuencia, **los documentos D1 y D4 no hacen que la presente invención sea obvia.**

Finalmente, como se ha explicado a lo largo del trámite de la solicitud, la presente invención proporciona efectos sorprendentes.

En este sentido, se hace referencia a la Tabla 1 de la descripción. En el grupo de control de toxicidad, el BUN ($87,8 \pm 6,6$ mg/dl) y la creatinina ($1,13 \pm 0,17$ mg/dl) séricos aumentaron significativamente en comparación con el grupo de control normal ($18,7 \pm$

2,5 mg/dl y $0,29 \pm 0,04$ mg/dl) ($p < 0,005$), lo que indica que la función renal estaba dañada en el grupo de control de toxicidad.

En el grupo de prueba, la naringenina, el kaempferol y la luteolina solos mostraron BUN ($36,0 \pm 7,8$ mg/dl) ($31,5 \pm 7,6$ mg/dl a $58,8 \pm 7,0$ mg/dl) ($23,7 \pm 4,4$ mg/dl a $31,5 \pm 7,3$ mg/dl) y creatinina ($0,32 \pm 0,03$ mg/dl) ($0,38 \pm 0,09$ mg/dl a $0,70 \pm 0,09$ mg/dl) ($0,25 \pm 0,07$ mg/dl a $0,43 \pm 0,08$ mg/dl) séricos; y Eudragit S100, Pluronic F68, Sucralosa y Manitol solos mostraron BUN ($24,4 \pm 2,8$ mg/dL), ($29,1 \pm 3,4$ mg/dL), ($33,6 \pm 2,7$ mg/dL), ($41,0 \pm 2,6$ mg/dL) y creatinina ($0,32 \pm 0,07$ mg/dL), ($0,37 \pm 0,13$ mg/dL), ($0,40 \pm 0,15$ mg/dL), ($0,56 \pm 0,13$ mg/dL) séricos.

Sorprendentemente, la naringenina, el kaempferol o la luteolina, en combinación con Eudragit S100 y Pluronic F68, mostraron efectos mejorados en la recuperación de la función renal, **muy cerca o incluso mejores que un nivel normal**, en comparación con los compuestos solos.

Específicamente, una combinación de Naringenina, Pluronic F68 y Eudragit S100 mostró el BUN ($20,3 \pm 4,1$ mg/dL) y creatinina ($0,22 \pm 0,04$ mg/dL) séricos, una combinación de Kaempferol, Manitol y Sucralosa mostró el BUN ($16,2 \pm 2,8$ mg/dL) y creatinina ($0,18 \pm 0,03$ mg/dL) séricos, una combinación de Naringenina, Manitol y Sucralosa mostró el BUN ($15,0 \pm 3,0$ mg/dL) y creatinina ($0,19 \pm 0,03$ mg/dL) séricos, y una combinación de Eudragit S100, sucralosa y Luteolina mostró el BUN ($16,7 \pm 2,8$ mg/dL) y creatinina ($0,19 \pm 0,03$ mg/dl) séricos, **muy cerca o incluso mejor que un nivel normal**.

En consecuencia, la presente invención proporciona una combinación particular de compuestos que tienen efectos superiores en la prevención, reducción o erradicación de la nefrotoxicidad causada por APAP, que no es predecible a partir de las referencias citadas.

Teniendo en cuenta lo anteriormente expuesto, queda claro que las características de la materia reclamada en el capítulo reivindicatorio presentado junto al presente memorial no se derivan de manera obvia o evidente de las enseñanzas de D1 y D4. Por lo tanto, la presente solicitud cumple con el requisito de nivel inventivo estipulado en el artículo 18 de la Decisión 486.

7. Conclusiones

Con las modificaciones introducidas y los argumentos presentados puede concluirse que:

- i) El nuevo título de la solicitud es claro y se relaciona con el objeto reclamado, cumpliendo así con el literal d) del artículo 27 de la Decisión 486 de 2000;
- ii) Las modificaciones del nuevo pliego reivindicatorio se ajustan a las disposiciones del Artículo 34 de la Decisión 486;
- iii) La invención de la presente solicitud es clara y cuenta con el debido sustento en el capítulo descriptivo, a la vez que se encuentra divulgada de manera suficientemente completa, cumpliendo así con los requerimientos de los Artículos 30 y 28 de la Decisión 486;
- iv) La invención reivindicada no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica por una persona del oficio normalmente versada en la materia técnica, cumpliendo así con el requerimiento del Artículo 18 de la Decisión 486.

Por tanto, atentamente me permito solicitar a ese Despacho otorgar el privilegio de patente solicitado a la solicitud en estudio, toda vez que cumple a cabalidad con los requerimientos de la Decisión 486 de 2000.

Asimismo, respetuosamente solicito a ese Despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional.

8. Anexos

- Nuevo capítulo reivindicatorio que incluye cinco (5) reivindicaciones.
- Recibo de pago por concepto de modificación al pliego reivindicatorio.
- Anexo 1: Artículo *Canadian journal of physiology and pharmacology*, 88(10), 960-967.
- Anexo 2: Artículo *Drug Development and Industrial Pharmacy*, 41(11), 1793-1800.

Señora Directora,



CAROLINA DAZA MONTALVO

C.C. 66.783.790 de Palmira Valle

TP-141563 CSJ

notificaciones@clarkemodet.com.co

REIVINDICACIONES

1. Una composición que comprende:

un ingrediente activo, que comprende naringenina, kaempferol o luteolina, en combinación con poli(ácido metacrílico-metacrilato de metilo), copolímero en bloque de polioxietileno-polioxipropileno, manitol y/o sucralosa; y

un vehículo farmacéuticamente aceptable,

en donde la composición comprende de 0.1% a 100% en peso del ingrediente activo, en base al peso total de la composición.

2. La composición de acuerdo con la reivindicación 1, en donde el ingrediente activo incluye una combinación de poli(ácido metacrílico-metacrilato de metilo), copolímero en bloque de polioxietileno-polioxipropileno y naringenina.

3. La composición de acuerdo con la reivindicación 1, en donde el ingrediente activo es una combinación seleccionada del grupo que consiste en:

- una combinación de copolímero de poli(ácido metacrílico-metacrilato de metilo), sucralosa y luteolina;
- una combinación de kaempferol, manitol y sucralosa; y
- una combinación de naringenina, manitol y sucralosa.

4. La composición de cualquiera de las reivindicaciones 1 a 3, que además comprende una cantidad terapéuticamente efectiva de APAP.

5. La composición de cualquiera de las reivindicaciones 1 a 3, en la que cada ingrediente activo está incluido en la composición en una cantidad de 1 a 200 mg.

Influence of small doses of various drug vehicles on acetaminophen-induced liver injury

Tomislav Kelava, Ivan Čavar, and Filip Čulo

Abstract: The biological effects of drug vehicles are often overlooked, often leading to artifacts in acetaminophen-induced liver injury assessment. Therefore, we decided to investigate the effect of dimethylsulfoxide, dimethylformamide, propylene glycol, ethanol, and Tween 20 on acetaminophen-induced liver injury. C57BL/6 male mice received a particular drug vehicle (0.6 or 0.2 mL/kg, i.p.) 30 min before acetaminophen administration (300 mg/kg, i.p.). Control mice received vehicle alone. Liver injury was assessed by measuring the concentration of alanine aminotransferase in plasma and observing histopathological changes. The level of reduced glutathione (GSH) was assessed by measuring total nonprotein hepatic sulfhydryls. Dimethylsulfoxide and dimethylformamide (at both doses) almost completely abolished acetaminophen toxicity. The higher dose of propylene glycol (0.6 mL/kg) was markedly protective, but the lower dose (0.2 mL/kg) was only slightly protective. These solvents also reduced acetaminophen-induced GSH depletion. Dimethylformamide was protective when given 2 h before or 1 h after acetaminophen administration, but was ineffective if given 2.5 h after acetaminophen. Ethanol at the higher dose (0.6 mL/kg) was partially protective, whereas ethanol at the lower dose (0.2 mL/kg) as well as **Tween 20 at any dose had no influence**. None of the vehicles (0.6 mL/kg) was hepatotoxic per se, and none of them was protective in a model of liver injury caused by D-galactosamine and lipopolysaccharide.

Key words: acetaminophen, dimethylsulfoxide, dimethylformamide, propylene glycol, ethanol, Tween 20, liver injury.

Résumé : Les effets biologiques des excipients sont souvent négligés, ce qui peut causer des artéfacts dans l'évaluation d'une lésion hépatique induite par l'acétaminophène. Nous avons donc décidé d'examiner l'effet du diméthylsulfoxyde, du diméthylformamide, du propylèneglycol, de l'éthanol et du Tween 20 sur ce type de lésion. Des souris mâles C57BL/6 ont reçu un excipient spécifique (0,6 ou 0,2 mL/kg, i.p.) 30 min avant l'administration d'acétaminophène (300 mg/kg, i.p.). Les souris témoins n'ont reçu que l'excipient. La lésion hépatique a été évaluée en tenant compte de la concentration d'alanine aminotransférase dans le plasma et des modifications histopathologiques. Le taux de glutathion réduit (GSH) a été déterminé en mesurant les sulfhydryles hépatiques non protéiques totaux. Le diméthylsulfoxyde et le diméthylformamide (aux deux doses) ont presque totalement supprimé la toxicité de l'acétaminophène. Le propylèneglycol a eu un effet protecteur significatif à la dose élevée (0,6 mL/kg), mais un effet négligeable à la faible dose (0,2 mL/kg). Ces solvants ont aussi réduit la déplétion de GSH induite par l'acétaminophène. Le diméthylformamide a eu un effet protecteur lorsqu'il a été administré 2 h avant et 1 h après l'acétaminophène, mais n'a eu aucun effet lorsqu'il a été administré 2,5 h après l'acétaminophène. L'éthanol a eu un effet protecteur partiel à la forte dose, mais aucun à la faible dose. Le Tween 20 n'a pas eu d'effet. Aucun des excipients (0,6 mL/kg) n'a été hépatotoxique et aucun n'a eu un effet protecteur dans un modèle de lésion hépatique causée par la D-galactosamine et le lipopolysaccharide.

Mots-clés : acétaminophène, diméthylsulfoxyde, diméthylformamide, propylèneglycol, éthanol, Tween 20, lésion hépatique.

[Traduit par la Rédaction]

Introduction

Acetaminophen (APAP) is a widely used analgesic and antipyretic drug that is considered safe at therapeutic doses. Overdose of APAP causes liver damage and for the last 2 decades is the leading cause of acute hepatic failure in the Western world (Lee 2004; Norris et al. 2008). The toxicity of APAP is initiated by formation of a reactive metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), by liver microso-

mal enzymes; CYP1A2 and CYP2E1 seem to be the most important isoforms in mice (in humans, CYP2E1 and CYP3A4 appear to be the most important isoforms). NAPQI first depletes glutathione (GSH) and then covalently binds to cellular proteins (Mitchell et al. 1973; Nelson 1982; Zaher et al. 1998; Laine et al. 2009). APAP-induced liver injury (AILI) in mice is the most extensively used model for study-

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T. Kelava.¹ Department of Physiology, School of Medicine, University of Zagreb, Šalata 3b, Zagreb 10000, Croatia.

I. Čavar. Department of Physiology, School of Medicine, University of Mostar, 88000 Mostar, Bosnia and Herzegovina.

F. Čulo. Department of Physiology, School of Medicine, University of Zagreb, Šalata 3b, Zagreb 10000, Croatia; Department of Physiology, School of Medicine, University of Mostar, 88000 Mostar, Bosnia and Herzegovina.

¹Corresponding author (e-mail: tkelava@mef.hr).

ing mechanisms of drug-induced liver injury (Gunawan and Kaplowitz 2007; Masson et al. 2008).

Some of the major biases in AILI investigations have occurred because the drug vehicles had biological effects per se, particularly dimethylsulphoxide (DMSO). For example, El-Hassan et al. (2003) reported a protective effect of the pancaspase inhibitor Z-VAD-fmk against APAP toxicity in mice. For controls, they used the cathepsin inhibitor benzylloxycarbonyl-Phe-Ala-fluoromethylketone (Z-FA-fmk) and achieved no protection with this compound. However, they did not add DMSO to Z-FA-fmk, which was used to dilute Z-VAD-fmk (El-Hassan et al. 2003). Subsequently, it was shown that DMSO and not Z-VAD-fmk protected mice from APAP (Jaeschke et al. 2006). Likewise, it was reported that natural killer cells have a pathogenic role in AILI (Liu et al. 2004), but recently it was shown that this happens only when DMSO is used to help dissolve APAP (Masson et al. 2008).

Commonly used vehicles in AILI experiments include DMSO (El-Hassan et al. 2003), ethanol (ETOH), corn oil (Donthamsetty et al. 2008), olive oil (Jodynis-Liebert et al. 2005), Tween 20 (Renić et al. 1993), Tween 80 (Kamanaka et al. 2003), propylene glycol (PG) (Oz and Chen 2008), and methylcellulose (Harrill et al. 2009). They are used for 2 purposes: (1) to facilitate solution of some other substance being investigated for its effect on AILI and (or) (2) to help dissolve APAP itself; in the latter case PG is frequently used. Despite its obvious importance, the influence of vehicles on AILI is rarely investigated. So far, it has been shown that DMSO, PG, and ETOH, if injected 15–30 min before or 1–2 h after APAP administration, reduce its toxicity, most probably by inhibition of cytochrome P450, which converts APAP to NAPQI. However, most of these investigations were conducted for doses of vehicles larger than 1 mL/kg (Hughes et al. 1991; Thomsen et al. 1995; Lee et al. 1999), which are 5–10 times higher than doses usually used in AILI experiments. A notable exception is DMSO, for which an effective dose-dependent study was conducted showing a strong protective effect even when DMSO was administered at a dose of 0.2 mL/kg (Yoon et al. 2006). The effects of various vehicles, to our knowledge, have not been compared.

Therefore, we decided to compare the effects of the 3 most frequently used solvents (DMSO, PG, and ETOH) on AILI, as well as to investigate the effects of Tween 20 and dimethylformamide (DMF), whose influence in this model have not been investigated. The main object of our study was to show which vehicles at doses of 0.2 and 0.6 mL/kg change the intensity of AILI and which do not. Besides common parameters of APAP toxicity, such as serum aminotransferase levels and liver pathohistology, the influence of APAP on GSH content in liver and microsomal enzyme activity was also investigated.

Materials and methods

Animals

C57BL/6 mice were raised in an animal colony unit at the Department of Physiology, School of Medicine, University of Zagreb, Zagreb, Croatia. Male mice aged 12–16 weeks and weighing 20–25 g were used in all experiments. The

cages were stored in rooms with a 12 h light period from 0600 to 1800, a temperature of 21 ± 2 °C, and a relative humidity of $50\% \pm 5\%$. The cages were sanitized twice weekly. All mice were given free access to tap water and standard mouse chow diet (Diete Standard 4 RF21 Mucedola S.r.l., Milan, Italy). All animal protocols followed the guidelines of and were approved by the Ethics Committee of the School of Medicine, University of Zagreb.

Chemicals

Pure APAP was a kind gift from the Belupo Pharmaceuticals and Cosmetics (Koprivnica, Croatia). Trichloroacetic acid, ETOH (96%), DMSO, PG, DMF, and phenobarbitone were purchased from Kemika, Zagreb, Croatia. Reduced L-glutathione, D-galactosamine (GalN), bovine serum albumin, and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (St. Louis, Mo., USA).

APAP-induced liver injury

A total of 5 experiments were conducted. In all experiments mice were fasted overnight (for 16 h). The next morning, mice were randomly divided into groups ($n = 6$ –8 animals per group) and received saline, DMSO, DMF, PG, or Tween 20. All solvents were diluted in saline to a final volume of 0.2 mL per animal and injected intraperitoneally at 0.6 mL/kg in the first experiment and 0.2 mL/kg in the second experiment. Thirty minutes later, APAP (i.p. at 300 mg/kg, in a volume of approximately 0.5 mL per animal), dissolved in warm saline (37 °C) under mild magnetic stirring, was administered. Six groups of animals received only vehicle (i.e., without APAP). Two types of control groups were included: mice that received saline before APAP administration and mice given saline alone.

The doses of 0.2 and 0.6 mL/kg were selected by searching the literature and manufacturers' data on the solubility of various substances, which were investigated previously for their effect on AILI, and solubility of APAP. It was found that effective doses of many substances, as well as APAP, can be dissolved with doses of solvents within this range. The data about solvents' toxicity were also examined; LD₅₀ for DMSO, DMF, PG, ETOH, and Tween 20 in mice is reported to be 12.6, 6.2, 9.3, 4, and 2.4 mL/kg (i.p.), respectively (Bartsch et al. 1976). It was also recommended not to use doses higher than a quarter of LD₅₀ for pharmacological and toxicological investigations, which gives 0.6 mL/kg as an upper limit for Tween 20. The preliminary toxicological study was also conducted for the dose of 0.6 mL/kg, and neither of the vehicles used caused toxicity in mice.

In a majority of published experiments, APAP and investigated substance were both injected intraperitoneally. To exclude the effect of the same administration route and possible interference of chemicals, we conducted the third experiment, which was almost identical to the first experiment except that APAP was now administered per os, by gastric lavage.

In the fourth experiment, saline or DMF (0.6 mL/kg, i.p.) was injected 2 h before or 1, 2.5, and 4 h after APAP administration.

In the fifth experiment, mice were given phenobarbitone (300 mg/L) in drinking water for 7 days prior to APAP administration, to induce liver microsomal enzymes. DMSO

(0.2 mL/kg, i.p.) or saline was injected 30 min before APAP administration.

In all experiments food was returned 4 h after APAP administration, and after an additional 3 h mice were anesthetized with Avertin; blood was collected by puncture of the medial eye angle with heparinized glass capillary tubes, and the livers were removed for analyses.

It is important to note that the doses of vehicles were adjusted to be equal in millilitres per kilogram (not in mg/kg or mol/kg). This was done deliberately, because the volume of the solvent is what is important when addition of a solvent is being considered.

Hepatic injury induced by GalN and lipopolysaccharide (LPS)

Mice were injected simultaneously with GalN (300 mg/kg, i.p.) and LPS (15 µg/kg, i.p.). Saline, DMSO, DMF, ETOH, PG, or Tween 20 was injected (0.6 mL/kg, i.p.) 30 min before GalN plus LPS treatment.

Evaluation of liver injury

Alanine aminotransferase (ALT) levels were determined from plasma by standard laboratory techniques in a clinical diagnostic laboratory. A portion of the liver was fixed in 10% neutral formalin, processed by histological techniques, stained with hematoxylin–eosin, and examined for morphological evidence of liver injury.

Total hepatic nonprotein sulfhydryl content determination

GSH level was assessed by measuring total hepatic non-protein sulfhydryls, using the method of Ellman (Ellman 1959). A portion (approximately 100 mg) of liver was homogenized in Tris–HCl buffer (25 mmol/L, pH 7.4), and 0.1 mL of the homogenate was mixed with 0.2 mL of 5% trichloroacetic acid, followed by centrifugation at 860g for 10 min. Supernatant was mixed with DTNB. The absorbance was determined at 412 nm. The absorbance was compared with a standard curve prepared using 6 different GSH concentrations. From the rest of the homogenate, the protein concentrations were determined using the Bradford method, using bovine serum albumin as the standard (Bradford 1976), and GSH content was calculated in nanomoles per milligram of protein.

Measurement of phenobarbitone sleeping time

Influence on duration of phenobarbitone sleeping time was determined as an in vivo measure of liver microsomal enzyme activity (Piel et al. 1969; Chan et al. 2009). Mice ($n = 4$ per group) were injected with saline, DMSO, DMF, PG, ETOH, or Tween 20 (0.6 mL/kg), and after 30 min they received phenobarbitone (50 mg/kg, i.p.). The sleeping time was defined as the sleep-time period from the loss of the righting reflex to its complete recovery (Piel et al. 1969; Henderson et al. 2003; Chan et al. 2009).

Statistical analysis

Data are presented as means $\pm$ SE. Statistical significance was evaluated by one-way ANOVA followed by Dunnett's multiple comparison tests. Results were considered statistically significant at $P < 0.05$.

Table 1. Effects of DMSO, DMF, ETOH, PG, and Tween 20, given at a dose of 0.6 mL/kg, on APAP-induced liver injury.

Vehicle (0.6 mL/kg)	APAP (mg/kg)	ALT (U/L)	GSH (nmol/ mg protein)
Saline	300	6747.5 $\pm$ 2290.4	9.8 $\pm$ 1.26
DMSO	300	178.6 $\pm$ 96.9*	53.2 $\pm$ 1.7*
DMF	300	135 $\pm$ 47.7*	56.1 $\pm$ 1.0*
ETOH	300	4633.8 $\pm$ 2066.4	19.6 $\pm$ 5.1
PG	300	441.4 $\pm$ 317.8*	46.2 $\pm$ 5.7*
Tween 20	300	5268.6$\pm$2512.6	9.4$\pm$2.5
Saline	—	20 $\pm$ 1.5	64.9 $\pm$ 0.5
DMSO	—	30.7 $\pm$ 5.7	65.8 $\pm$ 0.9
DMF	—	27 $\pm$ 1.5	57.1 $\pm$ 0.28 [†]
ETOH	—	32.7 $\pm$ 5.2	63.8 $\pm$ 1.3
PG	—	28.3 $\pm$ 4.4	65.1 $\pm$ 0.7
Tween 20	—	37.3 $\pm$ 7.1	64.5 $\pm$ 2.4

Note: Mice were treated with DMSO, DMF, ETOH, PG, Tween 20, or saline intraperitoneally 30 min before APAP administration (300 mg/kg, i.p.) or only with DMSO, DMF, ETOH, PG, Tween 20, or saline. GSH and ALT levels were determined 7 h after APAP. Each value represents the mean $\pm$ SE of 6–8 mice. GSH content was measured as total hepatic nonprotein sulfhydryl content. *, significantly different from mice treated with saline before APAP administration (ANOVA followed by Dunnett's multiple comparison test, $p < 0.05$); [†], significantly different from mice given only saline (ANOVA followed by Dunnett's multiple comparison test, $p < 0.05$). APAP, acetaminophen; ALT, alanine aminotransferase; GSH, reduced glutathione; DMSO, dimethylsulphoxide; DMF, dimethylformamide; ETOH, ethanol; PG, propylene glycol.

Results

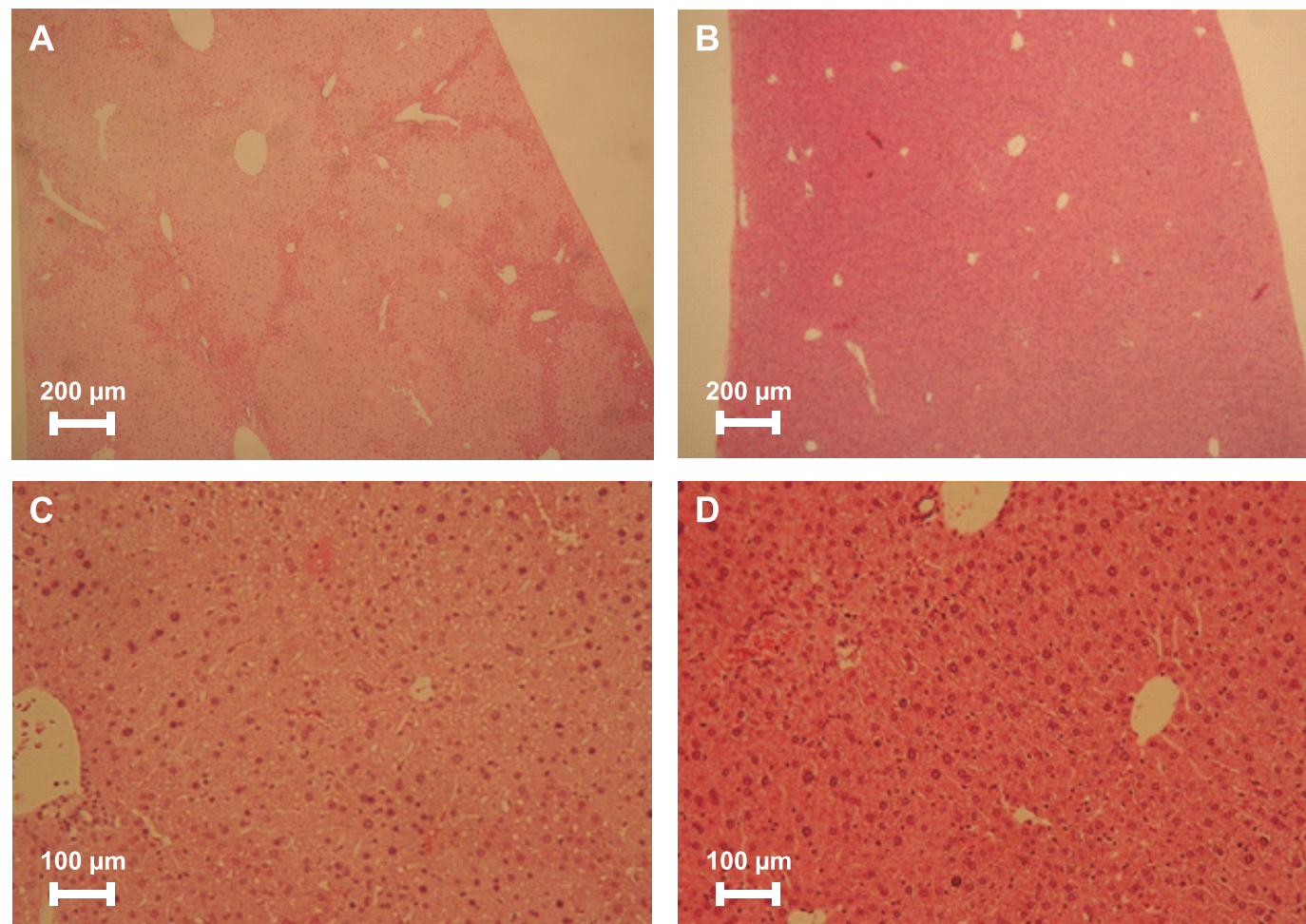
Effects of drug vehicles on APAP-induced liver injury

In the first experiment, drug vehicles were given 30 min before APAP administration (300 mg/kg, i.p.). As shown in Table 1, DMSO, DMF, and PG at a dose of 0.6 mL/kg almost completely abolished APAP toxicity; ALT levels were 37, 45, and 15 times lower, respectively, than in the control group (which received saline before APAP administration). ETOH only slightly reduced ALT levels, and Tween 20 had no influence. APAP depleted GSH from liver, and DMF, DMSO, and PG reduced this depletion. The protective effect of drug vehicles was clearly visible in histopathological analysis (Fig. 1); centrilobular necrosis, which was seen in mice given saline (or Tween 20) before APAP administration (Fig. 1A, 1C), was almost absent in mice given DMF (Fig. 1B, 1D) or DMSO before APAP administration.

When given alone (i.e., without APAP treatment), none of the vehicles had a significant effect on the ALT level; that is, the concentration of ALT in plasma was similar to that in plasma from mice given saline. However, DMF slightly reduced the GSH level in comparison to that in mice given saline, while other vehicles had no such effect (Table 1).

At a dose of 0.2 mL/kg (second experiment), DMF and DMSO were similarly effective; ALT levels were, respectively, 15 and 18 times lower than in the control group. They also significantly inhibited GSH depletion. At this dose, PG also reduced ALT concentration and blocked GSH depletion, but not significantly. ETOH and Tween 20 had no influence. DMF alone had no influence on GSH levels (Table 2).

Fig. 1. Effects of drug vehicles on histopathological changes in liver. Hematoxylin–eosin stained liver sections. (A, C) Typical liver sections (A: 100× original magnification; C: 200× original magnification) from a mouse treated with saline 30 min before APAP administration (300 mg/kg) showing extensive necrosis of centrilobular hepatocytes. The ALT level measured in the plasma of this mouse was 6480 U/L. Liver sections of mice treated with Tween 20 before APAP administration looked similar. (B, D) Typical liver sections (B: 100× original magnification; D: 200× original magnification) from a mouse treated with dimethylformamide (0.6 mL/kg) 30 min before APAP administration (300 mg/kg) showing essentially normal histology. The ALT level measured in the plasma of this mouse was 150 U/L. Liver sections of mice treated with PG or DMSO (0.6 mL/kg) before APAP treatment looked similar.



Protective effect of drug vehicles was not dependant on the administration route

In previous experiments, both vehicle and APAP were injected intraperitoneally, with short intervals between the 2 injections, which might cause physical or chemical interference of the agents. To exclude this, we conducted an experiment in which APAP was administered per os by gastric lavage. As shown in Fig. 2, the effect of solvents on concentration of ALT in plasma was very similar to that when APAP was given intraperitoneally (comparison of data in Table 1 and Fig. 2).

Protective effect of DMF was dependant on time of DMF application

To examine the effect of time, DMF was given at various time points before or after APAP administration. DMF completely blocked APAP toxicity if given 2 h before APAP administration; it was also effective if given 1 h after APAP

administration, but ineffective 2.5 or 4 h after APAP (Fig. 3).

Phenobarbitone pretreatment had no influence on protective effect of DMSO

It is known that phenobarbitone induces microsomal enzymes and therefore stimulates formation of a toxic metabolite of APAP, NAPQI (Belardinelli et al. 2008). Mice were pretreated for 7 days with phenobarbitone in drinking water (300 mg/L) and then received DMSO (0.2 mL/kg, i.p.) or saline prior to APAP (300 mg/kg, i.p.). As shown in Fig. 4, DMSO was also highly protective in this model (Fig. 4).

DMF and DMSO prolonged phenobarbitone sleeping time

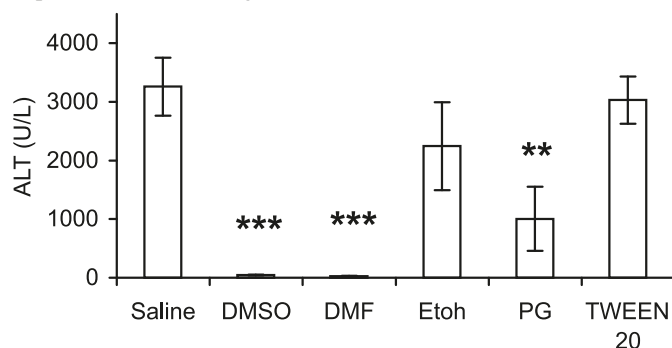
Sleeping time was measured in mice injected with saline or drug vehicles (0.6 mL/kg) 30 min before phenobarbitone administration (50 mg/kg, i.p.). DMSO and DMF significantly increased phenobarbitone sleeping time. PG pro-

Table 2. Effects of DMSO, DMF, ETOH, PG, and Tween 20, given at a dose of 0.2 mL/kg, on APAP-induced liver injury.

Vehicle (0.2 mL/kg)	APAP (mg/kg)	ALT (U/L)	GSH (nmol/mg protein)
Saline	300	8767±1663	9.7±0.9
DMSO	300	585±397**	44.9±1.7**
DMF	300	470±188**	44±2.9**
ETOH	300	8991±2048	12.1±4.5
PG	300	5830±1242	17.2±5.5
Tween 20	300	8774±1762	11±2.2
Saline	—	25.6±1.6	67.5±1.8
DMSO	—	29±1.3	65.6±0.5
DMF	—	34.6±3.5	65.7±0.7
ETOH	—	24.6±1.9	65.8±1.9
PG	—	20.3±0.2	65.3±1.8
Tween 20	—	29.6±3.3	64.1±2.1

Note: Mice were treated with DMSO, DMF, ETOH, PG, Tween 20, or saline intraperitoneally 30 min before APAP administration (300 mg/kg, i.p.) or only with DMSO, DMF, ETOH, PG, Tween 20, or saline. GSH and ALT levels were determined 7 h after APAP. Each value represents the mean ± SE of 6 mice. GSH content was measured as total hepatic nonprotein sulfhydryl content. **, significantly different from mice treated with saline before APAP administration (ANOVA followed by Dunnett's multiple comparison test, $p < 0.01$). APAP, acetaminophen; ALT, alanine aminotransferase; GSH, reduced glutathione; DMSO, dimethylsulphoxide; DMF, dimethylformamide; ETOH, ethanol; PG, propylene glycol.

Fig. 2. Effects of DMSO, DMF, ETOH, PG, and Tween 20 on concentration of ALT in plasma, when APAP was given orally. Mice were treated with DMSO, DMF, ETOH, PG, Tween 20, or saline (0.6 mL/kg, i.p.) 30 min before APAP administration (300 mg/kg, per os). ALT levels were determined 7 h after APAP administration. Each value represents the mean ± SE of 6 mice. ** and ***, significantly different from mice treated with saline before APAP administration (ANOVA followed by Dunnett's multiple comparison test; $p < 0.01$ and $p < 0.001$, respectively). DMSO, dimethylsulphoxide; DMF, dimethylformamide; ETOH, ethanol; PG, propylene glycol; ALT, alanine aminotransferase; APAP, acetaminophen; GSH, reduced glutathione.



longed it only slightly, while Tween 20 and ETOH had no influence (Table 3).

Effects of drug vehicles on liver injury induced by GalN and LPS

Treatment of mice with GalN and LPS highly increased plasma ALT levels (Fig. 5). None of the vehicles had a sig-

Fig. 3. Effect of varying time of DMF application on ALT plasma levels. Mice were treated with saline or DMF (0.6 mL/kg, i.p.) at indicated times prior to or after APAP (300 mg/kg, i.p.). ALT levels were determined 7 h after APAP. Each value represents the mean ± SE of 6 or 7 mice. * and **, significantly different from mice treated with saline and APAP (ANOVA followed by Dunnett's multiple comparison test; $p < 0.05$ and $p < 0.01$, respectively). DMF, dimethylformamide; ALT, alanine aminotransferase; APAP, acetaminophen.

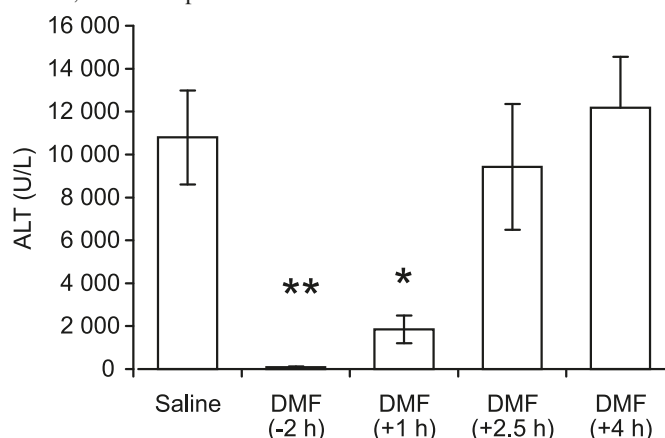
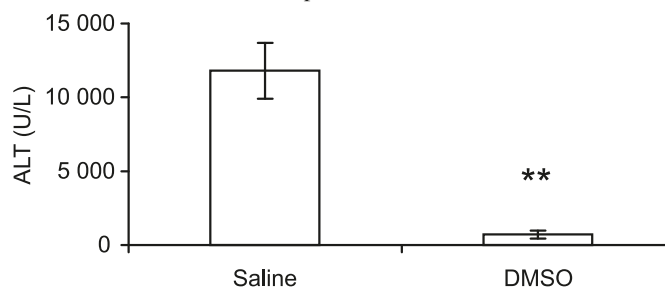


Fig. 4. Effect of DMSO on ALT levels in mice pretreated with phenobarbitone. Mice were pretreated for 7 days with phenobarbitone in drinking water (300 mg/L) and then treated with DMSO (0.2 mL/kg, i.p.) or saline prior to APAP (300 mg/kg, i.p.). ALT levels were determined 7 h after APAP administration. Each value represents the mean ± SE of 8 mice. **, significantly different from mice treated with saline before APAP administration (Student's t test, $p < 0.001$). DMSO, dimethylsulphoxide; ALT, alanine aminotransferase; APAP, acetaminophen.



nificant effect on the concentration of ALT, indicating that they are not protective against liver injury induced by LPS plus GalN.

Discussion

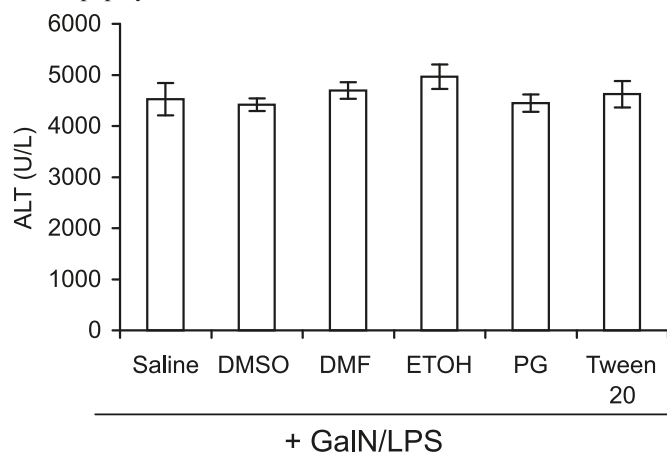
Although vehicles are generally considered relatively inert, they can induce biological effects that are often overlooked. AILI is shown to be very sensitive to bias caused by solvents (Jaeschke et al. 2006; Masson et al. 2008). Our results show that DMSO and DMF greatly suppress AILI even at small doses (0.2 mL/kg), PG is somewhat less protective, and it seems that doses of ETOH (0.2 mL/kg) and Tween 20 (0.2 and 0.6 mL/kg) have no effect. None of the vehicles appears to be hepatotoxic itself at the above-mentioned doses. The efficacy of vehicles in vivo was not dependent on the route of administration (Fig. 2); that is,

Table 3. Effects of DMSO, DMF, ETOH, PG, and Tween 20 (0.6 mL/kg) on phenobarbitone sleeping time.

Vehicle (0.6 mL/kg)	Sleeping time (min)
Saline	28.5±5.8
DMSO	60±5*
DMF	55.5±5.4*
ETOH	27±7.2
PG	34.5±6.3
Tween 20	26.7±3.5

Note: Mice were treated with DMSO, DMF, ETOH, PG, Tween 20, or saline intraperitoneally 30 min prior to phenobarbitone treatment. Each value represents the mean ± SE of 4 mice. *, significantly different from mice treated with saline (ANOVA followed by Dunnett's multiple comparison test, $p < 0.05$). DMSO, dimethylsulphoxide; DMF, dimethylformamide; ETOH, ethanol; PG, propylene glycol.

Fig. 5. Effects of DMSO, DMF, ETOH, PG, and Tween 20 on ALT levels in a GalN plus LPS model of liver injury. Mice were treated with DMSO, DMF, ETOH, PG, Tween 20, or saline (0.6 mL/kg, i.p.) 30 min prior to GalN plus LPS administration. ALT levels were determined 7 h after GalN plus LPS administration. Each value represents the mean ± SE of 6 mice. DMSO, dimethylsulphoxide; DMF, dimethylformamide; ETOH, ethanol; PG, propylene glycol; ALT, alanine aminotransferase; GalN, D-galactosamine; LPS, lipopolysaccharide.



DMSO, DMF, and PG were protective to a similar extent regardless of whether APAP was administered orally or intraperitoneally. The most probable means of protection is interference with the liver microsomal metabolism of APAP. This is supported with our findings that PG, DMSO, and DMF inhibited APAP-induced GSH depletion and prolonged phenobarbitone sleeping time. Yoon et al. proposed that DMSO inhibited microsomal formation of NAPQI in a competitive manner (Yoon et al. 2006). Therefore, we hypothesized that an experimental model in which microsomal enzymes are induced prior to APAP administration could be less sensitive to DMSO. However, our results showed that mice that received phenobarbitone for 7 days in drinking water (Guarner et al. 1988; Belardinelli et al. 2008) were equally protected by DMSO. Aside from its effect on APAP

metabolism, each of these vehicles can change several other parameters that play a role in AILI. For example, DMSO shows antioxidative activity (Jeffery et al. 1988; Dunphy et al. 2007), and several antioxidants protect against AILI (Marotta et al. 2009; Reisman et al. 2009). It also demonstrated adjuvant-like actions on the immune system (Kunze 1975; Yamasaki et al. 1988), which were the cause of bias in the previously mentioned natural killer cell investigation.

Because DMSO is so frequently a source of bias, we supposed that DMF might be a useful alternative (some of the substances that we intend to investigate in the future can be dissolved in both DMSO and DMF). However, DMF showed a protective effect similar to that of DMSO. There are no reports about the effect of DMF on oxidative stress or the immunological system. On the other hand, DMF shows anticoagulant-like effects (Imbriani et al. 1986), and some anticoagulants are able to protect from AILI (Ganey et al. 2007). Since this is the first report about the protective effect of DMF, we further investigated the effect of time of administration. It showed a typical pattern for substances that interfere with APAP metabolism, a very strong protection if given before or 1 h after APAP administration, but no protection if given 2.5 h after APAP administration, when biotransformation of APAP is essentially complete. DMF inhibits CYP2E1, most probably by suicidal enzyme inactivation (Tolando et al. 2001).

The protective effects of PG are well known; however, these were shown in experiments in which high doses of PG (2–4 mL/kg) were used (Hughes et al. 1991; Thomsen et al. 1995). Our results demonstrated that even small doses (0.2 mL/kg, and particularly 0.6 mL/kg) can suppress AILI. For ETOH, it is generally accepted that its chronic consumption increases AILI, but acute administration, together with or shortly before APAP administration, suppresses AILI (Prescott 2000). The dose of 0.2 mL/kg ETOH was not protective, and the dose of 0.6 mL/kg only slightly decreased ALT levels. PG and ETOH can reduce inflammatory responses and, opposite to the effect of DMSO, inhibit natural killer activity (Denning and Webster 1987; Oshiro et al. 1990; Chen et al. 2006; Pan et al. 2006).

Detergents such as Tween 20 and Tween 80 are also sometimes added to increase the solubility of lipophilic drugs in AILI experiments. It appears that Tween 20 at doses of 0.2 and 0.6 mL/kg has no influence on AILI or GSH content in the liver. There are no reports about its influence on the immunological response, oxidative stress, CYP2E1 activity, or coagulation system.

In another common model of liver injury, GalN plus LPS, none of the vehicles was protective or harmful. Interestingly, there are reports that show that DMSO is protective against GalN alone in rats. However, in those experiments the dose of DMSO was much higher (2.5 mL/kg) (Iida et al. 2007).

To avoid artifacts in AILI investigations it is advisable to use, when possible, pure saline or distilled water as a drug vehicle without adding any organic solvent or detergent. This is possible more frequently than is actually done. For example, 300 mg/kg of APAP, dissolved in warm saline under mild magnetic stirring, can be safely administered in a volume of approximately 0.5 mL per mice, and this is more than enough to induce AILI. Because PG and DMSO suppress AILI when used as vehicles for APAP, the dose of

APAP must be increased to 500 or even 750 mg/kg (Oz and Chen 2008) to cause similar liver damage. The result is an unnecessary increase in the APAP dose and application of a more biologically different organic solvent. Liu et al. reported that 300 mg/kg APAP is not enough to induce AILI in C57BL/6 mice and concluded that this strain was more resistant to APAP (Liu et al. 2004). Apparently, this was because DMSO was added to the APAP vehicle. In our experience, the 300 mg/kg dose of APAP induces severe hepatitis in CBA or BALB/c mice and as shown by Harill et al. in many other mouse strains (Harrill et al. 2009). When substances with low solubility in water are investigated for their effect on AILI, the control groups should be formed more carefully. Typical investigations include a treatment group, in which the substance of interest is dissolved in an organic vehicle, and the control group, in which only the vehicle is used. It would be good to include, at least in the first experiment, one more control group, in which only pure saline or distilled water is used, to better understand the effect of the vehicle. The dose of solvent or detergent added to the vehicle should be as small as possible and explicitly noted. It is probably better to use vehicles that interfere less with APAP toxicity. This means that Tween 20 and ETOH should be considered first, and then PG. The usage of DMSO or DMF should be limited to occasions when it is inevitable. Alternatively, some water-insoluble substances become soluble if the pH of the saline solution is changed. If some general hepatoprotective mechanism is investigated, it would be helpful to see whether it also protects against the GalN plus LPS model of hepatotoxicity, since this model appears to be more resistant to vehicle usage.

Finally, in the effort to avoid a possible experimental artifact with water-insoluble drugs, it is necessary to reduce the amount of injected biologically active organic solvent (or reduce its concentration in *in vitro* experiments) to the minimum dose possible, use an organic solvent with a smaller biological effect, or try to dissolve the substance in aqueous solvent with changed pH. Nonetheless, it is very important to include control groups in which the effect of the drug vehicle alone is tested.

Acknowledgements

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RESEARCH ARTICLE

Systemic exposure of Paracetamol (acetaminophen) was enhanced by quercetin and chrysin co-administration in Wistar rats and *in vitro* model: risk of liver toxicityRavindra Babu Pingili^{1,2}, A. Krishnamanjari Pawar³, and Siva R. Challa²

¹Research and Development, Department of Pharmacy, Jawaharlal Nehru Technological University, Kakinada, Andhra Pradesh, India, ²Department of Pharmacology, KVSRR Siddhartha College of Pharmaceutical Sciences, Vijayawada, Andhra Pradesh, India, and ³Department of Pharmaceutical Analysis, University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh, India

Abstract

Intestinal P-glycoprotein (P-gp) and drug-metabolizing enzymes (DMEs) play an important role in the first-pass-metabolism (FPM) and pharmacokinetics (PK) of majority of drugs. Paracetamol is primarily metabolized by conjugation reactions and a little amount (~15%) undergoes cytochrome P450 (CYP2E1)-mediated oxidative metabolism produces a hepatotoxic metabolite, *N*-acetyl-*p*-benzoquinonimine (NAPQI). Quercetin and chrysin are naturally occurring flavonoids, reported as modulators of P-gp and DMEs. Therefore, the objective of this study was to evaluate the effects of quercetin and chrysin on the pharmacokinetics of paracetamol using rats and non-everted gut sacs *in vitro*. Paracetamol was given orally (100 mg/kg) to rats alone and in combination with quercetin (5, 10 and 20 mg/kg) and chrysin (50, 100 and 200 mg/kg) once daily for 21 consecutive days. Blood samples were collected on the 1st day in single dose pharmacokinetic study (SDS) and on the 21st day in multiple pharmacokinetic studies (MDS). The plasma concentrations of paracetamol were determined by HPLC and PK parameters were calculated by using Kinetica (Version 5.1). The maximum plasma concentration (C_{max}) and area under the curve (AUC_{0-12}) of paracetamol was significantly increased by quercetin and chrysin co-administration in SDS and MDS. In non-everted rat gut sac method, the absorption of paracetamol was increased by presence of P-gp inhibitors (verapamil, quinidine and ketoconazole), quercetin and chrysin (50 µg/mL). Our findings suggested that the quercetin and chrysin might be inhibited the metabolism of paracetamol; thereby increased the systemic exposure of paracetamol and may be increased the risk of liver toxicity. Further studies are needed to evaluate whether the quercetin or chrysin are involved in the formation of NAPQI by CYP2E1 or not on isolated rat hepatocytes or using cell lines.

Keywords

CYP2E1, flavonoids, liver toxicity,
P-glycoprotein, pharmacokinetics

History

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Introduction

Paracetamol (acetaminophen) is a *para*-aminophenol derivative, possesses analgesic and antipyretic activities similar to aspirin. It is the preferred analgesic and antipyretic for patients in whom aspirin is contraindicated¹. It produces antipyretic effect by inhibiting prostaglandin synthesis in the brain². The analgesic effect of paracetamol is due to selective inhibition of cyclooxygenase (COX-2) dependent pathways³ and indirect activation of cannabinoid (CB1) receptors⁴. Paracetamol is primarily metabolized by glucuronidation and sulfate conjugation. It is also a substrate of a cytochrome P450 (CYP) CYP2E1, CYP1A2

and CYP3A4. The hepatotoxic metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI) is generated by the activation of CYP2E1 and CYP1A2 on its metabolism. It is a safe and effective drug but unintentional or intentional misuse is the number one cause of acute hepatic failure⁵. In addition, it undergoes significant first-pass-metabolism (FPM) and is a moderate substrate of P-glycoprotein (P-gp).

Quercetin, a flavonol has been shown to possess a wide spectrum of pharmacological effects including antioxidant, antinociceptive, anti-inflammatory, neuroprotective, anticancer⁶, hepatoprotective⁷ and nephroprotective activities⁸. Quercetin inhibits arachidonic acid pathway and platelet aggregation by inhibiting secretory phospholipase A₂ and cyclic phosphodiesterase, respectively⁹. Quercetin also suppresses cyclooxygenase (COX) COX-2 expression by inhibiting the p300 signaling and blocking the binding of multiple transactivators to COX-2 promoter¹⁰. Chrysin (5,7-dihydroxyflavone) is a naturally occurring flavonoid that exhibits many pharmacological effects, including antioxidant¹¹, anticancer, hepatoprotective¹² and nephroprotective¹³. Several studies reported that chrysin has

Address for correspondence: Siva Reddy Challa, Department of Pharmacology, KVSRR Siddhartha College of Pharmaceutical Sciences, Polyclinic Road, Siddhartha Nagar, Vijayawada, Andhra Pradesh 520010, India. Tel: +91-9885589543. Fax: 0866 2476086. E-mail: sivareddypharma@gmail.com; ravindrapingili@gmail.com

anti-inflammatory effect by inhibiting several cytokines, nitric oxide, prostaglandin E and COX-2¹⁴.

Moreover, quercetin and chrysin has been reported as modulators of P-gp and drug metabolizing enzymes. Therefore, the objective of this study was to investigate the effects of the quercetin and chrysin on the pharmacokinetics of paracetamol in rats and non-everted rat gut sacs (NEGS) *in vitro*.

Materials and methods

Drugs and chemicals

Quercetin and chrysin were purchased from Sigma Chemical Co. (St. Louis, MO). Paracetamol and verapamil were obtained as gift samples from Lancer Pharmaceuticals Pvt. Ltd, Baddi, India and Sipra Labs Ltd., Hyderabad, India, respectively. Ketoconazole and quinidine were obtained from Mylan Pharmaceuticals, Hyderabad. Methanol, acetonitrile and water of high-performance liquid chromatography (HPLC) grade were purchased from Finar Chemicals Ltd, Ahmadabad, India. All other chemicals and reagents used were of analytical grade.

Experimental animals

Animal experiments were performed according to the institutional guidelines for the care and use of laboratory animals, and approved by the animal ethics committee of KVSRR Siddhartha College of Pharmaceutical Sciences (SCOPS), Vijayawada, Andhra Pradesh, India (993/a/06/CPCSEA). Male Wistar rats (180–220 g) were procured from National Institute of Nutrition (NIN), Hyderabad, Andhra Pradesh, India. Animals were housed six per cage and given free access to food (Hindustan Lever, Mumbai, India) and water *ad libitum* in animal house at the KVSRR SCOPS. Before starting the experiments, animals were kept under standard laboratory conditions (12/12 h light/darkness, $22 \pm 2^\circ\text{C}$ and 50–60% humidity) for at least a week.

Experimental protocol

This study consists of a single dose pharmacokinetic study (SDS) and multiple dose pharmacokinetic study (MDS). In these studies, animals were treated with paracetamol alone and in combination with quercetin and chrysin once daily for 21 consecutive days, as it has been described previously by Venkatesh¹⁵. The doses of paracetamol, quercetin and chrysin for this study were calculated and selected based upon the observations from the earlier experiments. NEGS were used to determine the role of P-gp on the absorption of paracetamol.

Effect of quercetin on the pharmacokinetics of paracetamol in SDS and MDS

Five groups of male Wistar rats ($n=6$ per group), in a parallel design, received orally the following drugs once daily for 21 consecutive days:

Group I: Control group received 0.5 mL of vehicle (1% sodium carboxymethyl cellulose).

Group II: Paracetamol (100 mg/kg)¹⁶.

Group III: Paracetamol (100 mg/kg) + quercetin (5 mg/kg).

Group IV: Paracetamol (100 mg/kg) + quercetin (10 mg/kg)¹⁷.

Group V: Paracetamol (100 mg/kg) + quercetin 20 (mg/kg).

After treatment, 100 μL blood samples were collected from retro-orbital venous plexus¹⁸ at 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0 and 12.0 h under slight ether anesthesia on the 1st day in SDS and on the 21st day in MDS. The blood samples were centrifuged (Remi, R- 4C Compact model, Mumbai, India) at 6000 rpm for 10 min and plasma stored at -20°C until analysis.

Effect of chrysin on the pharmacokinetics of paracetamol in SDS and MDS

All Wistar rats were randomly divided into five groups of six animals in each group. The rats were treated orally with the following drugs once daily for 21 consecutive days:

Group I: Control group received 0.5 mL of vehicle (1% sodium carboxymethyl cellulose).

Group II: Paracetamol (100 mg/kg)¹⁶.

Group III: Paracetamol (100 mg/kg) + chrysin (50 mg/kg).

Group IV: Paracetamol (100 mg/kg) + chrysin (100 mg/kg)¹⁹.

Group V: Paracetamol (100 mg/kg) + chrysin (200 mg/kg).

After treatment, 100 μL blood samples were collected from retro-orbital venous plexus¹⁸ at 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0 and 12.0 h under slight ether anesthesia on the 1st day in SDS and on the 21st day in MDS. The blood samples were centrifuged (Remi, R- 4C Compact model, Mumbai, India) at 6000 rpm for 10 min and plasma stored at -20°C until analysis.

Extraction of paracetamol from plasma

A single step liquid–liquid extraction method was used to extract the paracetamol from the rat plasma²⁰. To an aliquot of 50 μL plasma, 1.5 mL ethyl acetate was added, vortex mixed for 5 min on a Remi vortex mixer and centrifuged at 3000 rpm for 5 min. The supernatant (1.4 mL) was dried and the dry residue was reconstituted in 200 μL of the mobile phase, then vortex-mixed. A 20- μL aliquot of the resulting solution was injected into HPLC system for analysis.

Analytical methods

The plasma concentrations of paracetamol were quantitated using a previously described method by Jan²¹ and Carmen with some modifications²². A Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisted of a pump (LC-20AT VP), C₁₈ column (Kromasil, 250 $\times$ 4.6 mm, 5 μ particle size) and a dual wavelength UV–visible detector (SPD-10A VP). Methanol: water (60:40, v/v) system was used as mobile phase and filtered through 0.45 μm membrane filter and ultrasonically (Hwashin Technology, Seoul, Korea) degassed before use. The injection volume was 20 μL and the effluent was monitored at 254 nm with a UV detector at a flow rate of 1 mL/min. The retention times of blank plasma and paracetamol were obtained at 2.868 and 3.367 min, respectively (Figure 1).

Method validation

Seven calibration samples (1, 2, 4, 6, 8, 10 and 20 $\mu\text{g/mL}$) were prepared by spiking blank plasma with appropriate volumes of the working solutions. Calibration curves were constructed between the concentration and peak area. Intra-day precision was defined by the percentage of relative standard deviation (RSD) of five standards at five different concentrations analyzed on the same day. Inter-day precision was estimated from the analysis of the five standards on five separate days during method validation.

Calculation of pharmacokinetic parameters

The plasma concentrations versus time data obtained from each individual rat were submitted to a non-compartmental pharmacokinetic analysis using Thermo Kinetica, Version 5.1 (Thermo Electron Corporation, Beverly, MA). The maximum plasma concentrations (C_{max}) and times to achieve maximum plasma concentrations (t_{max}) were obtained directly from the individual plasma concentration–time curves. Area under the plasma

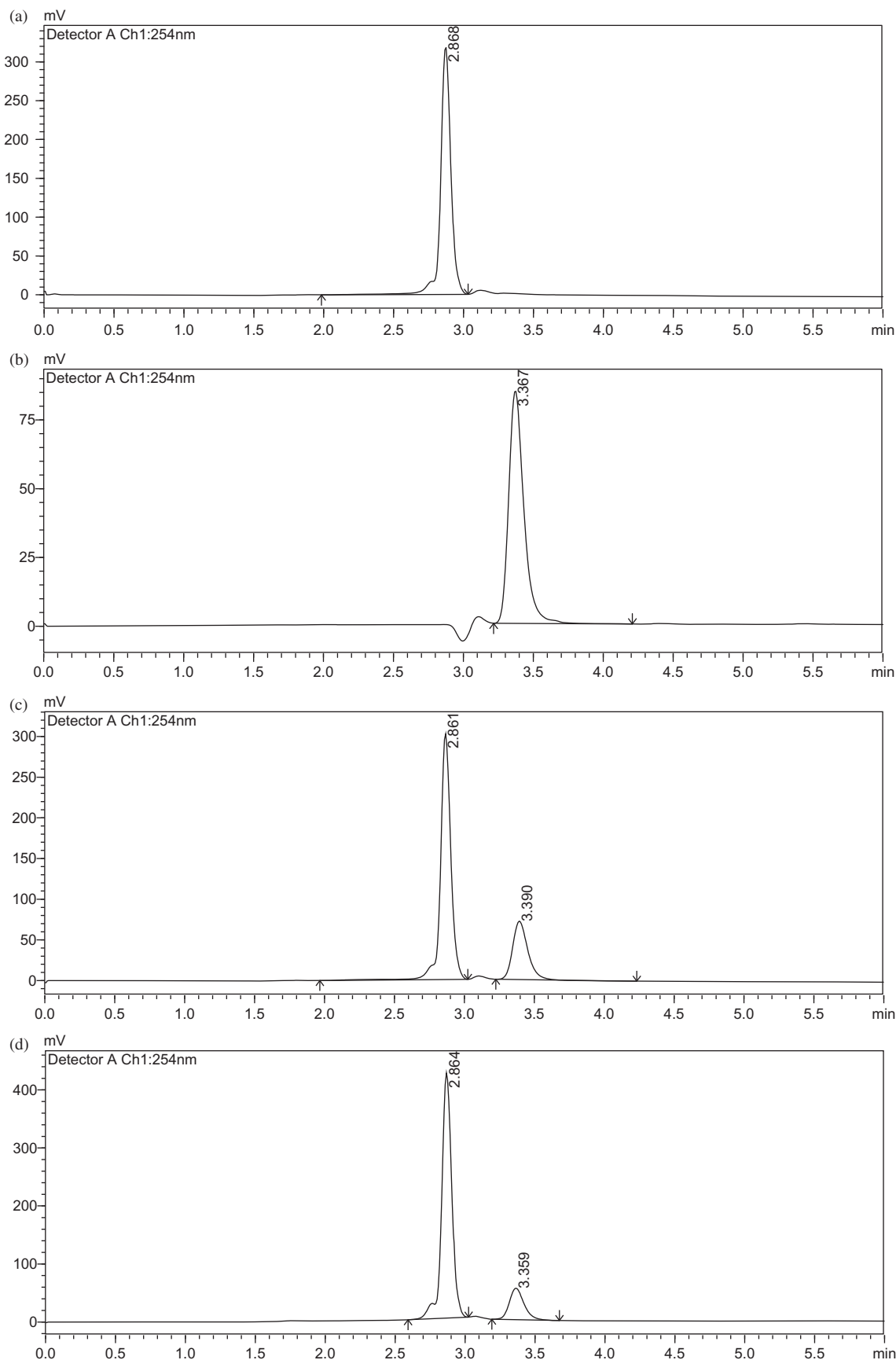


Figure 1. Representative chromatograms of (a) blank plasma (2.868); (b) paracetamol 4 µg/mL (3.367); (c) plasma (2.861) + paracetamol (3.390); (d) plasma sample obtained from rats treated with 100 mg/kg of paracetamol monitored at 254 nm.

concentration–time curve from time zero to 12 h (AUC_{0-12}) was calculated by the linear trapezoidal rule and $AUC_{0-\infty}$ was determined by the following formula:

$$AUC_{0-\infty} = C_{\text{last}}/K_{\text{el}} + AUC_{0-12},$$

where C_{last} is the last quantifiable concentration. The apparent total body clearance or oral clearance (CL/F) was calculated as follows:

$$CL/F = \text{dose}/AUC_{0-\infty}.$$

The elimination half-life ($t_{1/2}$) was calculated as $0.693/K_{\text{el}}$ and the mean residence time (MRT) was calculated as follows:

$$MRT = AUMC_{0-\infty}/AUC_{0-\infty}.$$

The area under the first moment curve ($AUMC_{0-\infty}$) was calculated from the plasma concentration–time curve. The volume of distribution (V_z/F) was estimated by means of $V_z/F = \text{dose}/AUC_{0-\infty}/K_{\text{el}}$. The apparent volume of distribution at steady state (V_{ss}/F) was estimated by means of $V_{ss}/F = CL \times MRT$. The results are presented as the mean $\pm$ standard deviation. The level of significance was set at $p < 0.05$.

Non-everted rat gut sac study *in vitro*

The everted and NEGS are simple and useful *in vitro* models to study the drug disposition. The NEGS were prepared using a method described previously by Ravindra with some modifications²³. Male Wistar rats weighing about 180–220 g were deprived of food for 1 day and provided with only double-distilled water before the experiments. The rats were anesthetized with pentobarbital sodium (40 mg/kg) and the small intestine was dissected out. The intestines were rinsed with ice cold saline in order to discard the intestinal digesta and the distal ileums (~10 cm each) were taken for the study.

Effect of quercetin and chrysin on paracetamol transport across non-everted gut sac

The NEGS were used to measure the transfer of paracetamol from mucosal to serosal compartment (M-to-S). NEGS were loaded with 1 mL of modified Krebs–Ringer bicarbonate (KRB) buffer containing paracetamol (100 $\mu\text{g}/\text{mL}$) in the presence or absence of P-gp inhibitors (verapamil, quinidine and ketoconazole) and quercetin (50 $\mu\text{g}/\text{mL}$). Each sac was placed in individual 50 mL Erlenmeyer flasks contain 20 mL of oxygenated (O_2/CO_2 ; 95:5) KRB and incubated at 37 °C for 60 min in a shaker bath. The transport of paracetamol from the M-to-S was measured by sampling 1 mL of the outer medium (replaced by 1 mL KRB buffer) at 10, 20, 30, 40, 50 and 60 min. Each experiment was triplicated and analyzed for paracetamol by HPLC. The same experiment was repeated with chrysin (50 $\mu\text{g}/\text{mL}$).

Data analysis

All statistics were calculated using Graph Pad Prism 5.0 software (San Diego, CA). Pharmacokinetic parameter values for groups were compared using analysis of variance with Tukey's and Dunnett's tests for multiple comparisons. Two-way ANOVA followed by Bonferroni post-tests to compare to each column to column. The p value less than 0.05 were considered significant.

Results

Effect of quercetin on the pharmacokinetics of paracetamol in SDS and MDS

The time–plasma profiles of paracetamol after oral administration of the paracetamol alone (100 mg/kg) and in combination with

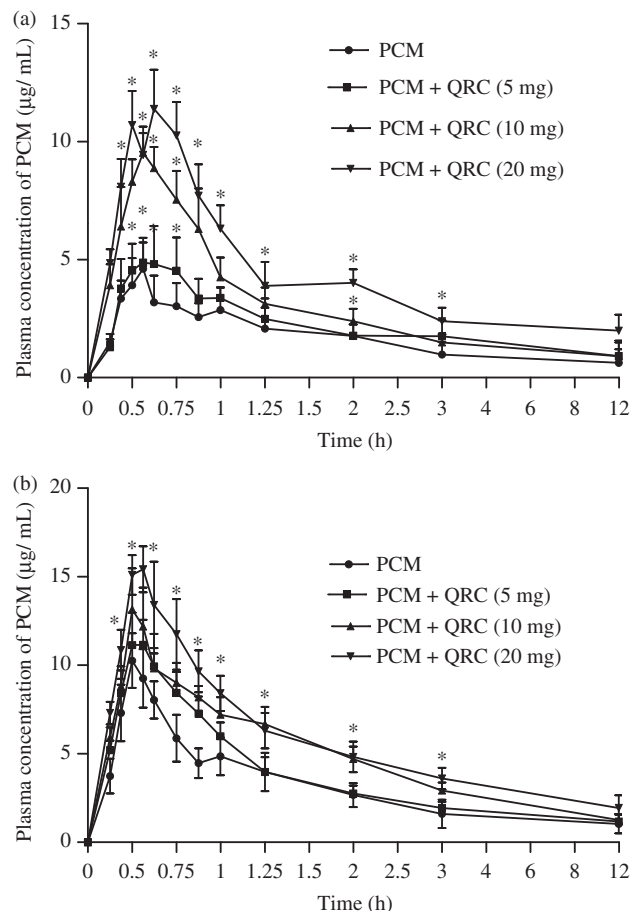


Figure 2. Mean plasma concentration–time profiles of paracetamol (100 mg/kg) following an oral administration of paracetamol to rats with or without quercetin (a) on 1st day; (b) on 21st day ($n = 6$). (●) Control (paracetamol), (■) with 5 mg/kg quercetin, (▲) with 10 mg/kg quercetin, (▼) with 20 mg/kg quercetin. All values are mean $\pm$ SD. Bars represent the standard deviation. * $p < 0.001$, ^{NS} $p > 0.05$ when compared to the paracetamol control group. PCM, paracetamol; QRC, quercetin (Two-way ANOVA followed by Bonferroni post-tests to compare to each column to column).

quercetin (5, 10 and 20 mg/kg) in SDS and MDS were shown in Figure 2. When paracetamol was co-administered with quercetin, the plasma concentrations of paracetamol were increased significantly compared to the paracetamol control (Figure 2). Mean pharmacokinetic parameters of paracetamol in plasma are presented in Tables 1 and 2. The C_{max} was significantly higher than those obtained when paracetamol was administered alone in both SDS and MDS. Treatment of rats with quercetin significantly increased the $AUC_{0-\infty}$ for paracetamol from 26.44 ± 6.79 to 64.47 ± 6.80 (quercetin 20 mg/kg, SDS) and 44.22 ± 10.66 to 81.49 ± 15.74 $\mu\text{g h}/\text{mL}$ (quercetin 20 mg/kg, MDS). The $t_{1/2}$ of paracetamol after quercetin treatments was longer than paracetamol control group. The results indicate that quercetin exerted the effect to inhibit the elimination of paracetamol and led to the elevation of C_{max} and $AUC_{0-\infty}$. The MRT of paracetamol was higher when this drug is administered with quercetin but it was statistically not significant. The CL/F , V_z/F and V_{ss}/F of paracetamol were significantly decreased by quercetin in SDS and MDS.

Effect of chrysin on the pharmacokinetics of paracetamol in SDS and MDS

The plasma concentration–time profiles of paracetamol after oral administration of 100 mg/kg paracetamol alone and in

Table 1. Pharmacokinetic parameters of paracetamol after the oral administration of paracetamol (100 mg/kg) to rats in the presence or absence of quercetin (5, 10 and 20 mg/kg, oral) on 1st day ($n = 6$).

Parameter	PCM (100 mg/kg)	PCM + QRC (5 mg/kg)	PCM + QRC (10 mg/kg)	PCM + QRC (20 mg/kg)
$C_{\max}$ ($\mu\text{g/mL}$)	5.04 ± 1.33	$5.83 \pm 0.79^{\text{NS}}$	$9.69 \pm 0.86^{***}$	$11.88 \pm 1.14^{***}$
AUC_{0-12} ($\mu\text{g h/mL}$)	19.71 ± 4.27	$25.68 \pm 5.85^{**}$	$36.04 \pm 1.69^{***}$	$51.41 \pm 3.96^{***}$
$\text{AUC}_{0-\infty}$ ($\mu\text{g h/mL}$)	26.44 ± 6.79	$33.89 \pm 9.19^{***}$	$41.39 \pm 5.16^{***}$	$64.47 \pm 6.80^{***}$
$t_{\max}$ (h)	1.16 ± 0.12	$1.41 \pm 0.34^{\text{NS}}$	$1.41 \pm 0.30^{\text{NS}}$	$1.33 \pm 0.40^{\text{NS}}$
$t_{1/2}$ (h)	4.43 ± 1.31	$4.96 \pm 1.74^{\text{NS}}$	$3.32 \pm 1.77^{\text{NS}}$	$4.39 \pm 0.77^{\text{NS}}$
MRT (h)	7.42 ± 2.25	$7.89 \pm 2.17^{\text{NS}}$	$5.58 \pm 1.76^*$	$7.19 \pm 1.43^{\text{NS}}$
CL/F (mL/h)	0.78 ± 0.17	$0.62 \pm 0.17^{\text{NS}}$	$0.48 \pm 0.06^{\text{NS}}$	$0.30 \pm 0.03^*$
V_z/F (mL)	4.91 ± 1.24	$4.27 \pm 1.05^{\text{NS}}$	$2.23 \pm 1.03^{***}$	$1.95 \pm 0.16^{***}$
V_{ss}/F (mL)	5.67 ± 1.32	$4.73 \pm 0.89^{\text{NS}}$	$2.64 \pm 0.58^{***}$	$2.21 \pm 0.28^{***}$

All values are mean $\pm$ SD. PCM, paracetamol; QRC, quercetin (Two-way ANOVA followed by Bonferroni post-tests to compare to each column to column).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $^{\text{NS}}p > 0.05$ when compared to the paracetamol control group.

Table 2. Pharmacokinetic parameters of paracetamol after the oral administration of paracetamol (100 mg/kg) to rats in the presence or absence of quercetin (5, 10 and 20 mg/kg, oral) on 21st day ($n = 6$).

Parameter	PCM (100 mg/kg)	PCM + QRC (5 mg/kg)	PCM + QRC (10 mg/kg)	PCM + QRC (20 mg/kg)
$C_{\max}$ ($\mu\text{g/mL}$)	10.66 ± 1.34	$11.91 \pm 1.45^{\text{NS}}$	$13.68 \pm 1.79^*$	$16.27 \pm 1.05^*$
AUC_{0-12} ($\mu\text{g h/mL}$)	37.59 ± 6.51	$44.90 \pm 3.62^*$	$58.33 \pm 4.88^{**}$	$68.27 \pm 7.87^{**}$
$\text{AUC}_{0-\infty}$ ($\mu\text{g h/mL}$)	44.22 ± 10.66	$53.32 \pm 5.90^*$	$64.67 \pm 4.47^{**}$	$81.49 \pm 15.74^{**}$
$t_{\max}$ (h)	1.08 ± 0.12	$1.16 \pm 0.20^{\text{NS}}$	$1.08 \pm 0.12^{\text{NS}}$	$1.20 \pm 0.18^{\text{NS}}$
$t_{1/2}$ (h)	3.82 ± 1.63	$4.66 \pm 1.95^{\text{NS}}$	$3.31 \pm 0.88^{\text{NS}}$	$4.34 \pm 1.23^{\text{NS}}$
MRT (h)	5.98 ± 1.89	$6.46 \pm 1.97^{\text{NS}}$	$5.47 \pm 0.95^{\text{NS}}$	$6.49 \pm 1.48^{\text{NS}}$
CL/F (mL/h)	0.47 ± 0.12	$0.37 \pm 0.04^{\text{NS}}$	$0.30 \pm 0.02^{\text{NS}}$	$0.24 \pm 0.04^*$
V_z/F (mL)	2.41 ± 0.577	$2.45 \pm 0.84^{\text{NS}}$	$1.47 \pm 0.38^{***}$	$1.52 \pm 0.19^{**}$
V_{ss}/F (mL)	2.67 ± 0.41	$2.37 \pm 0.54^{\text{NS}}$	$1.70 \pm 0.26^*$	$1.58 \pm 0.14^{**}$

All values are Mean $\pm$ SD. PCM, paracetamol; QRC, quercetin (Two-way ANOVA followed by Bonferroni post-tests to compare to each column to column).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $^{\text{NS}}p > 0.05$ when compared to the paracetamol control group.

combination with chrysin (50, 100 and 200 mg/kg) in SDS and MDS are illustrated in Figure 3. The non-compartmental pharmacokinetic parameters obtained after the oral administration of paracetamol alone and in the presence of 50, 100 and 200 mg/kg of chrysin are summarized in Tables 3 and 4. Statistical differences for $C_{\max}$ among paracetamol control group and chrysin co-administered groups were observed ($p < 0.001$). The $\text{AUC}_{0-\infty}$ of paracetamol increased from 27.98 ± 9.86 to 38.99 ± 5.96 , 44.58 ± 10.38 and $58.53 \pm 13.31 \mu\text{g h/mL}$ with 50, 100 and 200 mg/kg of chrysin, respectively, in SDS. Chrysin did not alter the MRT, $t_{\max}$ and $t_{1/2}$ of paracetamol significantly in SDS and MDS. The CL/F and V_z/F of paracetamol were significantly decreased by chrysin.

Effect of quercetin and chrysin on paracetamol absorption

To determine the role of P-gp on the transport of paracetamol, its transport activity was studied *in vitro* using gut sacs. This transport was increased in addition of verapamil, quinidine and ketoconazole to the serosal compartment with time in all groups studied (Figure 4). It is known that paracetamol may be transported or effluxed by P-gp and other ABC members present in the intestinal epithelium. To further confirm the specific participation of P-gp in paracetamol transport, repeated these same experiments, in the presence or absence of known P-gp inhibitors quercetin and chrysin (50 $\mu\text{g/mL}$). The results demonstrated that absorption of paracetamol was increased by quercetin and chrysin in a concentration-dependent manner at the end of the incubation period (Figure 4).

Discussion

According to World Health Organization, paracetamol is the cheapest and most widely used for the relief of the symptoms of rheumatoid arthritis, osteoarthritis, sprains, strains, sports injuries and menstrual disorders in the world²⁴. It is rapidly and almost completely absorbed from the gastrointestinal tract, primarily the small intestine by oral administration. Although the small intestine is regarded as an absorptive organ in the uptake of orally administered drugs, it also has the ability to metabolize drugs by numerous pathways involving both phase 1 and phase 2 reactions. Following usual oral doses, approximately 25% of paracetamol is metabolized on the first passage. Recent clinical studies have indicated that the small intestine contributes substantially to the overall first pass metabolism of many drugs like felodipine, carvedilol and metoprolol. Some studies have even suggested that the role of intestinal metabolism is quantitatively greater than that of hepatic metabolism in the overall first-pass effect²⁵. Paracetamol is metabolized by glucuronidation (40–67%) and sulfation (20–46%) into inactive and harmless metabolites. About 5–15% is oxidized by CYP2E1, CYP1A2, CYP3A4 and CYP2A6 resulting in the formation of the NAPQI. NAPQI is highly reactive as an electrophile and as an oxidant and can cause hepatic necrosis. Experimental evidence suggests that CYP2E1 is the most important isoform in paracetamol-induced hepatotoxicity. In the literature, not only the CYP2E1 is in relation to paracetamol activation and toxicity but also CYP1A2 is reported to be involved in the metabolic activation of paracetamol²⁶.

Hepatotoxicity due to overdoses of paracetamol has become an important problem worldwide. Use of herbal drugs in the

treatment of liver diseases has a long tradition, especially in Eastern medicine. Flavonoids have been shown to possess a variety of biological activities at non-toxic concentrations in organisms. Silymarin is a flavonoid, used exclusively for liver disease²⁷. The other flavonoids like quercetin and chrysin also used for liver diseases. For example, the manufacturer's recommended dose of chrysin is up to 6 capsules and one or multiple capsules of quercetin per day. Each capsule contains 500 mg.

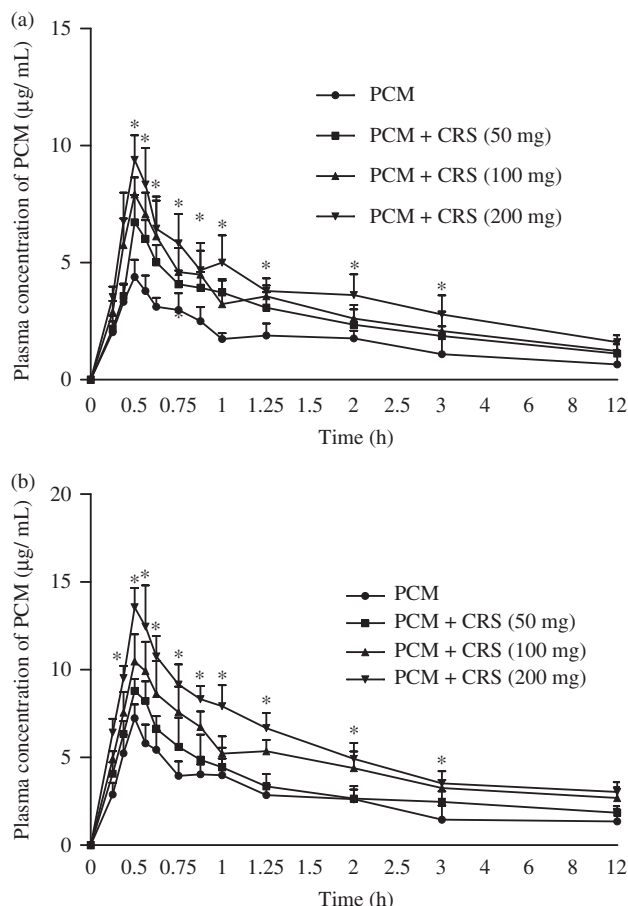


Figure 3. Mean plasma concentration–time profiles of paracetamol (100 mg/kg) following an oral administration of paracetamol to rats with or without chrysin (a) on 1st day; (b) on 21st day ($n = 6$). (●) Control (paracetamol), (■) with 50 mg/kg chrysin, (▲) with 100 mg/kg chrysin, (▼) with 200 mg/kg chrysin. All values are mean $\pm$ SD. Bars represent the standard deviation. * $p < 0.001$, ^{NS} $p < 0.05$ when compared to paracetamol control group. PCM, paracetamol; CRS, chrysin (Two-way ANOVA followed by Bonferroni post-tests to compare to each column to column).

Flavonoid–drug interactions have been increasingly observed due to the popular use of flavonoid-containing products for disease prevention and health promotion²⁸. However, our current understanding of the mechanisms underlying most of these interactions is related predominantly to the modulation of drug metabolizing enzymes and transporters. In order to better understand and predict the potential pharmacokinetic interactions of quercetin, chrysin with paracetamol disposition were investigated in this study.

The flavonoid quercetin has been reported to be potent inhibitor of the production of both sulfate and glucuronide conjugates of paracetamol in the cultured cells²⁹. Quercetin has been shown to increase the bioavailability of paclitaxel in rats in a concentration-dependent manner due to inhibition of P-gp, CYP2C9 and CYP3A4³⁰. In addition, quercetin was found to be a potent inhibitor of CYP1A2 and CYP2E1³¹. Shriner et al. reported that paracetamol glucuronidation is competitively inhibited by zidovudine thereby increased the plasma concentration of paracetamol in healthy volunteers³². Rutaecarpine induces the activities of hepatic CYP 1A2, 2B and 2E1 in rats. When the rats were pre-treated orally with rutaecarpine for 3 days, the percentage value of C_{max} and AUC of paracetamol-sulfate conjugate were significantly decreased. These results suggest that rutaecarpine might cause changes in the pharmacokinetic parameters of paracetamol in rats³³.

In this study, oral co-administration of quercetin with paracetamol significantly increased the AUC_{0–12} and C_{max} of paracetamol in SDS and MDS are shown in Tables 1 and 2. This study results and literature suggested that quercetin is an inhibitor of paracetamol metabolism thereby increased the AUC and C_{max} of paracetamol. Kamali et al. reported that pre-treatment with probenecid caused a significant decrease in paracetamol clearance in healthy volunteers due to inhibition of paracetamol metabolism³⁴. Oral co-administration of quercetin with paracetamol, dose-dependently decreased in paracetamol clearance. The other pharmacokinetic parameters like CL/F , V_z/F and V_{ss} of paracetamol decreased significantly with quercetin combination were shown in Tables 1 and 2. The paracetamol MRT and $t_{1/2}$ increased dose-dependently with quercetin co-administration in SDS and MDS.

The flavone chrysin has been reported to be potent inhibitor of glucuronidation and sulfation of paracetamol in the cultured cells²⁹. Chrysin is reported as a potent inhibitor of CYP1A2³⁵ and CYP2E1³⁶. Chrysin significantly increased the AUC and C_{max} of the oral paracetamol in both SDS and MDS (Tables 3 and 4). In rats, administration of chrysin decreased the clearance and $t_{1/2}$ of paracetamol in SDS and MDS. P-gp plays a significant role in drug absorption and disposition in animals and humans. Both CYP3A4 and P-gp function to protect the body from toxic

Table 3. Pharmacokinetic parameters of paracetamol after the oral administration of paracetamol (100 mg/kg) to rats in the presence or absence of chrysin (50, 100 and 200 mg/kg, oral) on 1st day ($n = 6$).

Parameter	PCM (100 mg/kg)	PCM + CRS (50 mg/kg)	PCM + CRS (100 mg/kg)	PCM + CRS (200 mg/kg)
C_{max} (µg/mL)	5.00 $\pm$ 1.27	7.01 $\pm$ 0.89 ^{NS}	7.94 $\pm$ 0.71 ^{NS}	9.48 $\pm$ 0.98**
AUC _{0–12} (µg h/mL)	19.14 $\pm$ 4.01	30.40 $\pm$ 2.41*	34.50 $\pm$ 6.14**	42.90 $\pm$ 5.51**
AUC _{0–∞} (µg h/mL)	27.98 $\pm$ 9.86	38.99 $\pm$ 5.96*	44.58 $\pm$ 10.38**	58.53 $\pm$ 13.31**
t_{max} (h)	1.08 $\pm$ 0.12	1.08 $\pm$ 0.12 ^{NS}	1.04 $\pm$ 0.10 ^{NS}	1.04 $\pm$ 0.10 ^{NS}
$t_{1/2}$ (h)	5.30 $\pm$ 2.90	4.84 $\pm$ 1.94 ^{NS}	5.34 $\pm$ 1.74 ^{NS}	6.13 $\pm$ 4.32 ^{NS}
MRT (h)	8.54 $\pm$ 4.03	7.92 $\pm$ 2.38 ^{NS}	7.99 $\pm$ 2.08 ^{NS}	9.46 $\pm$ 5.36 ^{NS}
CL/F (mL/h)	0.78 $\pm$ 0.24	0.51 $\pm$ 0.08 ^{NS}	0.47 $\pm$ 0.12*	0.35 $\pm$ 0.06**
V_z/F (mL)	5.28 $\pm$ 1.67	3.49 $\pm$ 0.91**	3.47 $\pm$ 0.82**	2.83 $\pm$ 1.21***
V_{ss}/F (mL)	5.97 $\pm$ 1.34	4.00 $\pm$ 0.67**	3.63 $\pm$ 0.69***	3.08 $\pm$ 0.96***

All values are Mean $\pm$ SD. PCM, paracetamol; CRS, chrysin (Two-way ANOVA followed by Bonferroni post-tests to compare to each column to column).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ^{NS} $p > 0.05$ when compared to the paracetamol control group.

Table 4. Pharmacokinetic parameters of paracetamol after the oral administration of paracetamol (100 mg/kg) to rats in the presence or absence of chrysin (50, 100 and 200 mg/kg, oral) on 21st day ($n = 6$).

Parameter	PCM (100 mg/kg)	PCM + CRS (50 mg/kg)	PCM + CRS (100 mg/kg)	PCM + CRS (200 mg/kg)
$C_{\max}$ ($\mu\text{g/mL}$)	7.24 ± 0.77	$9.06 \pm 0.65^{\text{NS}}$	$11.23 \pm 0.74^{***}$	$14.09 \pm 1.20^{***}$
AUC_{0-12} ($\mu\text{g h/mL}$)	30.72 ± 4.47	$39.59 \pm 3.14^*$	$54.86 \pm 4.28^{***}$	$66.15 \pm 4.63^{***}$
$\text{AUC}_{0-\infty}$ ($\mu\text{g h/mL}$)	46.43 ± 10.44	$55.60 \pm 3.87^*$	$87.43 \pm 12.20^{***}$	$96.76 \pm 23.44^{***}$
$t_{\max}$ (h)	1.00 ± 0.0	$1.08 \pm 0.12^{\text{NS}}$	$1.08 \pm 0.12^{\text{NS}}$	$1.08 \pm 0.12^{\text{NS}}$
$t_{1/2}$ (h)	6.27 ± 2.67	$6.03 \pm 1.01^{\text{NS}}$	$8.25 \pm 1.88^{\text{NS}}$	$6.71 \pm 3.67^{\text{NS}}$
MRT (h)	9.83 ± 3.77	$9.38 \pm 1.12^{\text{NS}}$	$11.97 \pm 2.90^{\text{NS}}$	$10.16 \pm 4.23^{\text{NS}}$
CL/F (mL/h)	0.44 ± 0.09	$0.35 \pm 0.02^{\text{NS}}$	$0.22 \pm 0.03^{\text{NS}}$	$0.21 \pm 0.04^*$
V_z/F (mL)	3.76 ± 1.00	$3.17 \pm 0.61^{\text{NS}}$	$2.71 \pm 0.43^{**}$	$1.90 \pm 0.48^{***}$
V_{ss}/F (mL)	4.13 ± 0.94	$3.38 \pm 0.45^{\text{NS}}$	$2.71 \pm 0.34^{***}$	$2.04 \pm 0.29^{***}$

All values are Mean $\pm$ SD. PCM, paracetamol; CRS, chrysin (Two-way ANOVA followed by Bonferroni post-tests to compare to each column to column).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $^{\text{NS}}p > 0.05$ when compared to the paracetamol control group.

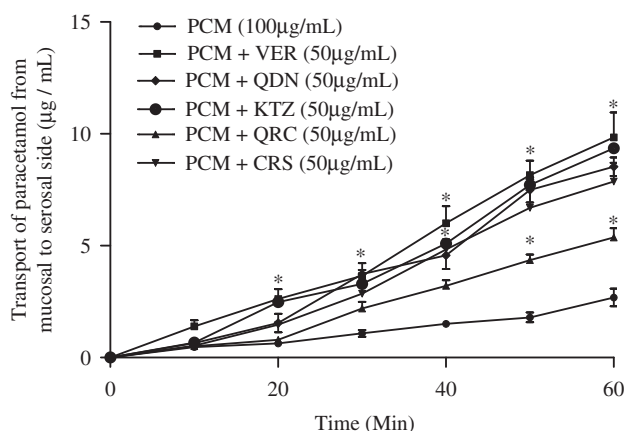


Figure 4. The transport of paracetamol (100 $\mu\text{g/mL}$) from mucosal to serosal side in the absence and presence of verapamil, quinidine, ketoconazole, quercetin and chrysin (50 $\mu\text{g/mL}$). PCM: paracetamol; VER: verapamil; QDN: quinidine; KTZ: ketoconazole; QRC: quercetin; CRS: chrysin. The data represent the mean $\pm$ SD ($n = 3$). * $p < 0.001$ significantly different compared to the paracetamol group.

accumulation of hydrophobic xenobiotics via metabolism and excretion. To determine the role of P-gp on the transport of paracetamol, studied its transport activity across non-everted gut sac *in vitro*. The absorption of paracetamol increased in presence of quercetin, chrysin and verapamil in a concentration-dependent manner across non-everted gut sacs. It is reported that paracetamol is a P-gp inducer at high doses³⁷. Carolina et al. reported that paracetamol is a P-gp inducer at the dose of 0.6 g/kg in rats³⁸. When compared to paracetamol therapeutic dose, the dose used in this study is very high. The maximum therapeutic dose of paracetamol is 4 g/day in humans³⁹. The dose of paracetamol is calculated based on the body weight of the animal. The selected dose of paracetamol is 100 mg/kg which is close to the therapeutic dose. The results demonstrate that absorption was increased by quercetin and chrysin in a concentration-dependent manner, at the end of the incubation period. Quercetin and chrysin are well known CYP2E1 inhibitors; thereby increased the absorption of paracetamol. This study results also revealed paracetamol may be a P-gp substrate at this dose.

Oxidative stress and NAPQI, hepatotoxic metabolite generated by CYP2E1 and CYP1A2 activation plays a major role in hepatic toxicity⁴⁰. Dietary flavonoids are natural antioxidants and potent inhibitors of CYP2E1 and CYP1A2. *In vitro* studies reveal that the flavonols (quercetin) and flavones (chrysin) has potent antioxidant activity, mainly based on scavenging of oxygen radicals⁴¹.

Conclusion

Inhibition and induction of P-gp and CYP enzymes have been reported as the causes of drug–drug or drug–food interactions. This study results suggest that quercetin and chrysin significantly increased the systemic exposure of paracetamol might be due to inhibition of metabolism and P-gp. Dosage adjustment is needed when paracetamol co-administered with either quercetin or chrysin. Further studies are needed to evaluate whether the quercetin or chrysin are involved in the formation of NAPQI by CYP2E1 or not on isolated rat hepatocytes or using cell lines.

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Declaration of interest

The authors declare that this research does not have any conflict of interest with anyone or any institute. This study was supported by Siddhartha Academy of General and Technical Education (SAGTE).

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**Superintendencia de
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CAROLINA MERCEDES DAZA MONTALVO
CC: 66.783.790
Consignación No: 1045073

Patente PCT

COMPOSICIONES QUE COMPRENDEN NARINGENINA, KAEMPFEROL O LUTEOLINA PARA REDUCIR LA TOXICIDAD DEL ACETAMINOFÉN (APAP)

Detalle del pago

Id.	Rentístico	Concepto y Servicio	Cant.	Val. Unit.	Subtotal
1	50005-01-03	Modificaciones / Correcciones al registro Patente PCT (DESCUENTO INTERNET)	1	\$ 214,000	\$ 214,000
TOTAL					\$ 214,000