UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

ANA PAULA DEL BORTOLO RUENIS

AVALIAÇÃO DE MODELOS FARMACOCINÉTICOS *IN VIVO* E *IN VITRO* PARA A ANÁLISE DE FÁRMACOS.

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, para obtenção do título de Doutor em Odontologia - Área de Farmacologia, Anestesiologia e Terapêutica do Programa de Pós-Graduação em Odontologia.

PIRACICABA
2004

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

ANA PAULA DEL BORTOLO RUENIS

FARMACÊUTICA

AVALIAÇÃO DE MODELOS FARMACOCINÉTICOS *IN VIVO* E *IN VITRO* PARA A ANÁLISE DE FÁRMACOS.

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, para obtenção do título de Doutor em Odontologia - Área de Farmacologia, Anestesiologia e Terapêutica do Programa de Pós-Graduação em Odontologia.

Orientador: Prof. Dr. Pedro L. Rosalen

Co-orientadores: Profa. Dra. Sílvia Storpirtis

Prof. Dr. Francisco Carlos Groppo

PIRACICABA
2004

Ficha Catalográfica

Ruenis, Ana Paula Del Bortolo.
R837a Avaliação de modelos farmacocinéticos *in vivo* e *in vitro* para a análise de fármacos. / Ana Paula Del Bortolo Ruenis. -- Piracicaba, SP : [s.n.], 2004.
xvi, 111p. : il.

Orientadores : Prof. Dr. Pedro Luiz Rosalen, Prof. Dr. Francisco Carlos Groppo, Profª Drª Sílvia Storpirtis.
Tese (Doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Farmacocinética. 2. Carbamazepina. 3. Farmacologia. I. Rosalen, Pedro Luiz. II. Groppo, Francisco Carlos. III. Storpirtis, Sílvia. IV. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. V. Título.

Ficha catalográfica elaborada pela Bibliotecária Marilene Girello CRB/8–6159, da Biblioteca da Faculdade de Odontologia de Piracicaba - UNICAMP.

DEDICATÓRIA

À minha mãe, Maria Aparecida, que através do seu amor infinito, seu exemplo de coragem e seu incondicional apoio às minhas escolhas, levou-me a alcançar todas as conquistas de minha vida.

Ao meu irmão, Paulo José, pelo carinho e apoio dispensados em todos os momentos.

Ao meu namorado, Rodrigo, pela paciência, compreensão, companheirismo e, principalmente, pelo seu amor, que me sustenta nas horas difíceis e dá sentido aos momentos felizes.

Ao meu pai, Antônio José, minha avó Laura, à minha avó Amábile (estes *in memoriam*) e aos meus familiares, por seu apoio e amor.

A Deus e a vocês, dedico este trabalho.

AGRADECIMENTOS ESPECIAIS

Ao meu orientador, Prof. Dr. Pedro Luiz Rosalen, por ter acreditado neste projeto e por seu apoio e orientação, cujos ensinamentos e exemplo de ética e profissionalismo têm norteado minha formação acadêmica e profissional.

Ao meu co-orientador, Prof. Dr. Francisco Carlos Groppo, idealizador deste projeto, por sua orientação, apoio, dedicação à pesquisa, sua disponibilidade de auxiliar em todos os momentos e sua amizade.

À Profa. Dra. Sílvia Storpirtis, da Faculdade de Ciências Farmacêuticas da USP, pelo exemplo de profissionalismo e oportunidade de aprendizado, que me incentivam a prosseguir nesta área.

Ao Prof. Dr. Leslie Z. Benet, da *University of California at San Francisco* (UCSF), por ter me recebido em seu laboratório e assim contribuído significativamente para minha formação científica.

> "Pela oportunidade de trabalharmos juntos e pelas amizades que daí surgiram, agradeço..."

Aos meus queridos amigos da FOP e de sempre: Simone, Juliana, Regiane, Giovana, Karla, Flávia, Roberta Baglie, Jéssica, Nilza, Sinvaldo, Gilson, Rogério, Ramiro, Patrícia, Fernando, Mirella, Adriana.

> "De cada um de vocês, levo comigo os risos, as brincadeiras, as conversas, os conselhos, a ajuda no trabalho, o apoio na distância, o companheirismo e a certeza de seu carinho e amizade."

AGRADECIMENTOS

À Universidade Estadual De Campinas, por meio do Reitor: Carlos Henrique De Brito Cruz.

À Faculdade De Odontologia De Piracicaba (FOP - UNICAMP), por meio de seu Diretor: Thales Rocha De Mattos Filho.

À Coordenação Geral dos Cursos de Pós-Graduação da FOP, por meio de seu Coordenador Lourenço Correr Sobrinho.

À Maria Cristina Volpato, Coordenadora do Programa Pós–Graduação em Odontologia.

Ao Departamento de Ciências Fisiológicas da FOP, por meio de seu Chefe de Departamento: Pedro Luiz Rosalen.

Ao CNPq, Conselho Nacional de Desenvolvimento Científico e Tecnológico, pela concessão da bolsa de estudos que permitiu a realização deste trabalho.

À CAPES, Centro de Aperfeiçoamento de Pessoal Especializado, pela concessão de bolsa de estudo do Programa de Doutorado com Estágio no Exterior (PDEE), para realização do estágio na UCSF.

A Sinvaldo Baglie, Gilson César Nobre Franco, Roberta Pessoa Simões, Luiz Madaleno Franco e Gláucia Maria Bovi Ambrosano, amigos e companheiros do Centro de Estudos em Biodisponibilidade e Bioequivalência, termos juntos concretizado a implantação do projeto do Centro.

Aos Professores da Área de Farmacologia, Anestesiologia e Terapêutica da FOP/UNICAMP, Maria Cristina Volpato, Thales Rocha de Mattos Filho, Eduardo Dias De Andrade e José Ranali pela convivência e pelos ensinamentos dispensados.

A Ronilson A. Moreno e Eduardo Abib Júnior, da Synchrophar Assessoria e Desenvolvimentos de Projetos Clínicos, pela oportunidade de desenvolvimento deste trabalho.

À Maria Elisa dos Santos, secretária eficientíssima da Área de Farmacologia, Anestesiologia e Terapêutica, pela sua dedicação.

À Eliane Melo Franco de Souza e José Carlos Gregório, técnicos da Área de Farmacologia, Anestesiologia e Terapêutica, por seu auxílio inestimável.

À Érica Alessandra Pinho, secretária da Coordenadoria de Pós-Graduação da FOP-UNICAMP, pela solicitude e presteza de seus serviços.

A todos os colegas do Curso de Pós-Graduação em Odontologia da FOP/UNICAMP e do Laboratório do Prof. Leslie Z. Benet, em especial à Dhaval Desai e Frances Peterson, pela amizade, e Nobuaki Watanabe, pelos ensinamentos.

Aos amigos e pessoas, que direta ou indiretamente, embora não citados neste momento, me incentivaram e apoiaram durante o desenvolvimento deste trabalho.

SUMÁRIO

RESUMO

O objetivo geral deste trabalho foi o estudo de modelos farmacocinéticos *in vitro* e *in vivo*. Para tal, o presente trabalho foi conduzido em quatro etapas com objetivos específicos para cada modelo utilizado, tendo os fármacos carbamazepina e claritromicina como referência para estes estudos. Os estudos 1 e 2 avaliaram, respectivamente, a biodisponibilidade comparativa da carbamazepina e da claritromicina através de modelo de estudo de bioequivalência entre medicamentos teste e referência. Os parâmetros de eficácia da claritromicina na redução da microbiota oral e nasal de voluntários saudáveis e sua correlação com os dados farmacocinéticos obtidos no estudo 2 foram avaliados no estudo 3. No estudo 4, os efeitos interativos de enzimas metabólicas e transportadores celulares na disposição da claritromicina foram estudados. Os medicamentos teste e referência de carbamazepina e claritromicina foram considerados bioequivalentes em seus respectivos estudos. A claritromicina mostrou-se eficaz na redução da microbiota oral e nasal de voluntários saudáveis e, embora não tenha havido correlação significativa entre os parâmetros PK/PD e a redução de microrganismos, a utilização do modelo apresentado foi capaz de predizer melhor *in vivo* a eficácia da claritromicina, quando comparado aos modelos *in vitro* tradicionais. A claritromicina demonstrou ser um substrato para os efeitos de primeira-passagem intestinal mediados pela P-gp e CYP3A4, no modelo *in vitro* com células CYP3A4-Caco-2, e novos estudos envolvendo diferentes transportadores são necessários à completa elucidação deste mecanismo. Assim, os modelos farmacocinéticos *in vitro* e *in vivo* apresentados neste trabalho são adequados para avaliar aspectos importantes da disposição do fármaco no organismo, sendo aplicáveis tanto nos estágios iniciais de desenvolvimento de fármacos como na melhor compreensão das propriedades farmacocinéticas daqueles já registrados e comercializados.

Palavras-chave: farmacocinética, bioequivalência, CYP3A4, P-gp, claritromicina, carbamazepina.

ABSTRACT

The overall aim of this study was the evaluation of *in vitro* and *in vivo* pharmacokinetic models. The present study was conducted in four phases with specific objectives in each model, and the drugs carbamazepine and clarithromycin were used as reference for these studies. The studies 1 and 2 evaluated, respectively, the comparative bioavailability of carmabazepine and clarithromycin, using the bioequivalence model to verify differences between test and reference formulations. Clarithromycin efficacy parameters on the reduction of oral and nasal microbiota and their correlation with the pharmacokinetic data obtained in the study 2 were analysed in the study 3. In the study 4, the interactive effects of metabolic enzymes and cellular transporters on the disposition of clarithromycin were evaluated. Both clarithromycin and carbamazepine test and reference formulations were considered bioequivalents, in their respectives studies. Clarithromycin was effective in reducing oral and nasal microbiota of healthy volunteers and, although no significant correlation between the PK/PD parameters and the decrease in the bacterial counts were observed, this model was a better predictor of the *in vivo* efficacy of clarithromycin, when compared to traditional *in vitro* models. Iit was demostrated that clarithromycin is a substrate to the intestinal first-pass extraction mediated by P-gp and CYP3A4, in the *in vitro* CYP3A4-Caco-2 cells model, and further studies envolving different transporters are required to completely elucidate this mechanism. The pharmacokinetic *in vitro* and *in vivo* models presented in this study are suitable to evaluate important aspects of drug disposition and efficacy, and could be applied not only in the early stages of drug development, but also in the comprehension of the pharmacokinetic properties of registered drugs already available in the market.

Key-words: pharmacokinetics, bioequivalence, CYP3A4, P-gp, clarithromycin, carbamazepine.

1 INTRODUÇÃO

A integração entre os conceitos de biofarmácia, farmacocinética e farmacodinâmica permite a compreensão dos fenômenos decorrentes da exposição de um determinado organismo ao fármaco. Estas áreas do conhecimento consideram, respectivamente, as fases de liberação e dissolução do fármaco, os fenômenos que determinam a variação da concentração do fármaco nos fluidos corporais em função do tempo, e o efeito ou resposta terapêutica.

A crescente utilização de conceitos farmacocinéticos/farmacodinâmicos pode otimizar o processo de desenvolvimento de um novo medicamento, ou ainda racionalizar a utilização daqueles já existentes. Durante o estágio pré-clínico, modelos animais podem ser utilizados para a avaliação preditiva de potência, atividade intrínseca, toxicidade e dosagem. Na fase clínica, modelos farmacocinéticos/farmacodinâmicos permitem caracterizar a relação dose-concentração-efeito/toxicidade, avaliar os efeitos da idade e sexo, interações do fármaco com alimentos, outros fármacos e a patologia, desenvolvimento de tolerância, e as variabilidades inter e intraindividual (Meibohm & Derendorf, 2002).

Tem sido amplamente discutida a aplicação de conceitos farmacocinéticos/farmacodinâmicos na utilização racional de antimicrobianos, uma vez que a manutenção de concentrações séricas e teciduais em função do tempo é essencial para atingir-se a eficácia terapêutica. Não há consenso quanto ao melhor método preditivo de atividade antibacteriana dentre os modelos *in vitro* e *in vivo* utilizados até então (Turnidge, 1998; Li, 2000; Amsden, 2001; Van Bambeke & Tulkens, 2001; Fridt-Moller, 2002).

A aplicabilidade de modelos farmacocinéticos é bastante ampla. No Brasil, estudos de biodisponibilidade/bioequivalência de medicamentos ganharam maior destaque a partir da implantação da Lei 9.787/99 e Resolução 391/99 que estabeleceram o medicamento genérico no país (Brasil, 1999b). O objetivo destes dispositivos legais foi o de implantar uma política de medicamentos no país e instituir o medicamento genérico. Os estudos de biodisponibilidade e bioequivalência no Brasil seguem uma regulamentação técnica semelhante àquela dos Estados Unidos e Europa, a fim de assegurar a qualidade,

eficácia e segurança destes medicamentos, garantindo sua intercambialidade com o medicamento referência (Serra, 1998).

A bioequivalência entre medicamentos similares, em humanos, pode ser afetada tanto por diferenças farmacotécnicas no preparo das formulações quanto pelas biodisponibilidades individuais dos sujeitos da pesquisa. As características próprias do fármaco e de sua liberação, a partir da forma farmacêutica, exercem notável influência na quantidade e na velocidade da absorção. Assim, os fatores que afetam a desintegração, dissolução e conseqüentemente a biodisponibilidade de fármacos são resumidamente classificados em fatores físico-químicos, fatores relacionados à forma farmacêutica e seus excipientes e fatores fisiológicos que afetam o trato-gastrointestinal (Serra, 1998).

O conhecimento acerca dos mecanismos de transporte através da parede intestinal aumentou consideravelmente nos últimos anos. Tem sido demonstrada a existência de um mecanismo de proteção contra substâncias exógenas diferente do efeito de primeira passagem hepática (Benet & Cummins, 2001).

Benet & Cummins (2001), revisaram os principais mecanismos pelos quais a atividade do citocromo P450 e a glicoproteína P (P-gp) poderiam interferir no transporte e metabolismo de fármacos, utilizando células Caco-2 (linhagem de células de carcinoma de cólon humano) modificadas para expressar estas estruturas. Segundo estes autores, o fármaco é absorvido através de um processo passivo pelo enterócito, podendo ser metabolizado pelo CYP3A4, o principal sistema de biotransformação de fase I intestinal.

Entretanto, o fármaco também sofre co-transporte ativo pela P-gp, isto é, retorna ao lúmen intestinal e é reabsorvido, sendo exposto novamente à ação enzimática. Assim o papel da P-gp no intestino vai além da simples limitação da absorção do composto original, aumentando o acesso do fármaco ao metabolismo pelo CYP3A através de ciclos repetidos de absorção e efluxo, o que pode acarretar na diminuição de sua biodisponibilidade (Benet & Cummins, 2001).

Diante destes aspectos, o objetivo geral deste trabalho foi o estudo de modelos farmacocinéticos *in vitro* e *in vivo*, através de diferentes ensaios. Para tal, o presente trabalho foi conduzido em quatro etapas com objetivos específicos para cada modelo

utilizado, tendo os fármacos carbamazepina e claritromicina como referência para estes estudos.

2 REVISÃO DA LITERATURA

2.1 Modelo de estudo de biodisponibilidade e bioequivalência de medicamentos.

A partir da década de sessenta, o termo Biofarmacotécnica ou Biofarmácia definiu a área da Farmacologia que estuda os fatores físico e físico-químicos relacionados ao fármaco em sua forma farmacêutica, e a influência desta sobre a liberação do fármaco no organismo (Wagner, 1961). Os conceitos de Biofarmacotécnica relacionam a biodisponibilidade com o perfil físico-químico da formulação farmacêutica, ou seja, com as características de liberação e dissolução do fármaco (Storpirtis, 1993; Serra, 1998).

O conceito de Biodisponibilidade surgiu no início da década de 70, com a publicação de um estudo relatando diferenças entre as curvas de decaimento plasmático obtido após a administração de formulações contendo digoxina (Porta, 1999). Nesta década, relatos de episódios de intoxicação com digoxina decorrentes da alteração da biodisponibilidade de comprimidos de digoxina aumentaram o interesse por esses estudos, além de promoverem a divulgação dos conceitos relativos a biodisponibilidade e bioequivalência de medicamentos. Diversos estudos foram publicados, envolvendo fármacos como tetraciclinas, fenilbutazona, fenitoína, diazepam, e penicilinas (Porta, 1999).

A partir dessas constatações, o Food and Drug Administration (FDA-USA) promoveu estudos que resultaram na regulamentação dos critérios relativos a biodisponibilidade e bioequivalência de medicamentos (Storpirtis & Consiglieri, 1995; Braga, 2000).

Segundo o FDA, a biodisponibilidade indica a velocidade e extensão pelas quais um fármaco é absorvido a partir de uma forma farmacêutica e torna-se disponível no sítio de ação (United States, 1992). Definição semelhante foi adotada pela EMEA, European Agency for the Evaluation of Medicinal Products, em 1998; entretanto, este órgão considera que a substância na circulação sistêmica está em contínua troca com a substância no local de ação e, assim sendo, pode-se definir que a biodisponibilidade refere-se à extensão e à velocidade pelas quais um fármaco é liberado da forma farmacêutica para a circulação sistêmica (Porta, 1999).

O termo bioequivalência é definido pelo FDA (United States, 1992) como sendo a ausência de diferença significativa na velocidade e extensão pelas quais o fármaco presente em equivalentes farmacêuticos ou alternativas farmacêuticas torna-se disponível no local de ação, quando administrado na mesma dose molar e nas mesmas condições, em ensaio adequadamente planejado.

O FDA define ainda que os equivalentes ou alternativas farmacêuticas que apresentem diferença intencional em relação à velocidade podem ser consideradas bioequivalentes, caso não exista diferença significativa na extensão pela qual o fármaco torna-se disponível no local de ação. Este critério é válido quando se tratar de diferença intencional entre as velocidades de absorção, desde que esta diferença não interfira na obtenção de concentrações eficazes durante o uso crônico e não seja clinicamente significativa (United States, 1992).

Para a EMEA, dois produtos farmacêuticos (equivalentes ou alternativas farmacêuticas) são considerados bioequivalentes quando suas respectivas biodisponibilidades, após administração de mesma dose molar, forem de tal forma semelhantes que garantam os mesmos efeitos em relação à eficácia e segurança (Porta, 1999).

A ANVISA/MS (Agência Nacional de Vigilância Sanitária, Ministério da Saúde) define que o termo biodisponibilidade indica a velocidade e a extensão de absorção de um princípio ativo em uma forma de dosagem, a partir de sua curva concentração/tempo na circulação sistêmica ou sua excreção na urina. No Brasil, a Lei n° 9.787 de 10/02/1999 alterou a Lei n° 6.360 de 23/09/1976 e estabeleceu o medicamento genérico em nosso país (Brasil, 1999b).

O medicamento genérico, segundo a ANVISA/MS, é definido como medicamento similar a um produto de referência ou inovador, que se pretende ser com este intercambiável, geralmente produzido após a expiração ou renúncia da proteção patentária ou de outros direitos de exclusividade, comprovada a sua eficácia, segurança e qualidade, e designado pela DCB ou, na sua ausência, pela DCI (Brasil, 1999a).

A quase totalidade dos ensaios de bioequivalência é realizada em voluntários considerados saudáveis através de avaliação clínica e laboratorial, com a quantificação do

fármaco em fluidos biológicos como o plasma e urina. Os parâmetros farmacocinéticos são obtidos das curvas de concentração sangüínea ou urinária do fármaco *versus* tempo (Storpirtis & Consiglieri, 1995).

Os protocolos de bioequivalência devem ser elaborados de forma a reduzir a variabilidade inerente aos mesmos, de forma que quaisquer diferenças detectadas entre os parâmetros farmacocinéticos possam ser atribuídas aos medicamentos em estudo (Porta, 1999). São ensaios do tipo aberto, aleatório, cruzado, em dois períodos, cujas coletas devem contemplar o período correspondente a no mínimo três a cinco meias-vidas de eliminação do fármaco. O período de intervalo (*washout*) entre os dois períodos é estabelecido em no mínimo cinco a sete meias-vidas de eliminação (Brasil, 2001). Para a obtenção de resultados confiáveis, que permitam predizer com segurança os parâmetros que levarão à determinação de bioequivalência entre medicamentos, o ensaio de bioequivalência deve ser conduzido usando-se método analítico exato, preciso e reprodutível.

No caso de formas farmacêuticas orais de liberação convencional, os principais parâmetros farmacocinéticos a serem determinados são ASC_{0-t}, ASC_{0-inf} C_{max}, T_{max} (obtidos sem interpolação de dados), $T_{1/2}$, V*ad* e *Clearance*. Considera-se que os parâmetros ASC_{0-t}, C_{max} e T_{max} são os mais adequados para descrever a extensão e a velocidade de absorção de um fármaco (Brasil, 2001).

Desta forma, os órgãos regulatórios exigem evidências de biodisponibilidade média relativa expressa como razão entre as médias dos referidos parâmetros farmacocinéticos dos produtos teste e referência, utilizando-se dados logaritmicamente transformados. Os medicamentos serão considerados bioequivalentes quando IC de 90% estiver compreendido entre 80 e 125% (Brasil, 2001).

A fim de implementar o modelo de estudo de biodisponibilidade e bioequivalência na Área de Farmacologia, Anestesiologia e Terapêutica da Faculdade de Odontologia de Piracicaba/UNICAMP, foram utilizados, como referência, os fármacos claritromicina e carbamazepina. Os estudos foram realizados segundo a Resolução RDC nº 84, a Resolução - RE nº 479, e a Resolução - RE nº 478, todas de 19 de março de 2002 (Brasil, 2002).

A carbamazepina (CBZ) é uma das drogas mais utilizadas para o tratamento da epilepsia, sendo também comumente indicada para o tratamento da neuralgia do trigêmio e desordens psiquiátricas, particularmente a depressão bipolar (Mashford *et al.*, 1974; Spina *et al.*, 1996). Trata-se de um fármaco de baixa solubilidade em meio aquoso, e sua absorção gastrintestinal é caracterizada como lenta, irregular e incompleta, com $T_{1/2}$ elevada (Jung, 1997).

A claritromicina é um antimicrobiano do grupo dos macrolídeos que, na terapêutica odontológica, substituiu a eritromicina como alternativa às penicilinas, em pacientes com histórico de hipersensibilidade (Dajani *et al*., 1997; Taubert & Dajani, 1998), na profilaxia da endocardite infecciosa. Para este fármaco ainda foram aplicados modelos farmacocinéticos/farmacodinâmicos e *in vitro*, descritos a seguir.

2.2 Modelos farmacocinéticos/farmacodinâmicos aplicados ao estudo de antimicrobianos: o exemplo da claritromicina

A terapêutica antimicrobiana tem como objetivos a erradicação efetiva de microrganismos patogênicos, com a menor dose possível em curto período de duração de tratamento, e ocorrência mínima de efeitos adversos. A determinação de esquemas posológicos apropriados depende do conhecimento e integração das propriedades farmacocinéticas e farmacodinâmicas do antimicrobiano (Ebert & Craig, 1990).

As propriedades farmacodinâmicas dos antimicrobianos devem refletir a relação entre concentração e atividade antimicrobiana e/ou toxicidade. Segundo Ebert & Craig (1990), os parâmetros farmacodinâmicos mais freqüentemente utilizados para caracterizar a atividade antimicrobiana são a determinação da concentração inibitória mínima (CIM ou MIC, do inglês *minimum inhibitory concentration*) e a concentração bactericida mínima (CBM ou MBC, do inglês *minimum bactericidal concentration*).

Turnidge (1998) afirmou que a determinação da CIM seria o único parâmetro *in vitro* capaz de mensurar a atividade intrínseca dos antimicrobianos, uma vez que possibilita obter uma medida simples de concentração diretamente comparável às concentrações no sangue, fluídos corporais e tecidos. No entanto, é importante lembrarmos que a CIM é uma

medida *in vitro* que envolve a determinação subjetiva de turbidez em caldo, quando um patógeno isolado é exposto a diluições seriadas (1:2) do antimicrobiano por 18 a 24 h, a 35-37°C. Mesmo ensaios que utilizam difusão em ágar, como o Etest (Biodisk, Sweden) podem ter seus resultados influenciados por variáveis como densidade do inóculo, composição do meio, leitura do teste, entre outras (Fridt-Moller, 2002).

Em 1990, Ebert & Craig já consideravam que as determinações de CIM e CBM (concentração bactericida mínima, ou seja, a menor concentração que produz redução maior que 99,99% dos microrganismos viáveis) seriam insuficientes para a avaliação de eficácia antimicrobiana, pois refletem um fenômeno "tudo ou nada" (crescimento ou não-crescimento, erradicação ou não-erradicação) ocorrido após um período fixo de incubação e, desta forma, não consideram o efeito do antimicrobiano em função do tempo.

Outros indicadores de eficácia antimicrobiana são os estudos *time kill* (Credito *et al*., 1999; Visalli *et al.*, 1997). Uma curva *time kill* típica é gerada quando determinado patógeno é exposto a concentrações constantes do antimicrobiano, sendo constituída por uma fase de decaimento, uma fase log-linear de redução exponencial e uma fase de retorno do crescimento bacteriano. Uma fase estática pode ser observada quando subpopulações bacterianas resistentes atingem um nível de saturação devido ao total consumo de nutrientes do meio de cultura (Li, 2000). Neste método, a fase log-linear tem sido considerada o mais importante indicador da atividade antimicrobiana. A ligação das moléculas do fármaco com seu sítio de ação resulta em redução do número de microrganismos viáveis, ou seja, no efeito farmacológico esperado; assim, as constantes de redução bacteriana são saturáveis em altas concentrações.

Embora os estudos *time kill* forneçam informações mais detalhadas sobre a dinâmica da redução bacteriana frente ao antimicrobiano, o fato de que *in vivo* as concentrações do fármaco são alteradas durante o intervalo entre as dosagens não é contemplado por estes estudos. Assim, é necessária a incorporação de parâmetros farmacocinéticos aos dados obtidos *in vitro* a fim de ampliar a compreensão da atividade clínica de um antimicrobiano (Ebert & Craig, 1990; Van Bambeke & Tulkens, 2001).

Dentre os fatores farmacocinéticos que influenciam o resultado clínico final estão o C_{max}, a $T_{1/2\beta}$ e a quantidade total do antimicrobiano presente durante o período entre

doses, representada pelo parâmetro ASC_{0-t}. Outro fator importante é a ligação às proteínas plasmáticas: considera-se que se menos de 70% do antimicrobiano estiver ligado a proteínas, esta ligação não será clinicamente importante (Montgomery, 1991).

A necessidade de manutenção de concentrações plasmáticas acima da CIM para β-lactâmicos está baseada na observação de que uma taxa máxima de erradicação bacteriana pode ser atingida com concentrações plasmáticas ligeiramente superiores a CIM, um efeito concentração-independente (Li, 2000). De forma semelhante aos β-lactâmicos, a vancomicina e as tetraciclinas também exibem efeito concentração-independente e, nestes casos, não necessitam atingir picos de concentrações plasmáticas, representadas pelo parâmetro C_{max}. O parâmetro mais adequado parece ser a %T>CIM, ou seja, a porcentagem de tempo em que a concentração plasmática excede a CIM (Van Bambeke & Tulkens, 2001).

Por outro lado, o efeito bactericida de quinolonas, aminoglicosídeos metronidazol e anfotericina apresenta baixo grau de saturação em doses crescentes; desta forma maiores concentrações resultam em aumento do efeito antimicrobiano. Este efeito concentração-dependente é caracterizado pelos parâmetros C_{max}/CIM e ASC_{0-24}/CIM (Li, 2000; Van Bambeke & Tulkens, 2001).

A investigação da influência dos parâmetros farmacocinéticos sobre o efeito dos antimicrobianos *in vivo* tem sido reportada na literatura científica (Turnidge, 1998; Li, 2000; Amsden, 2001; Van Bambeke & Tulkens, 2001; Fridt-Moller, 2002; Toutain *et al*., 2002; Bonapace *et al*., 2002).

No caso dos macrolídeos, a determinação isolada de %T>CIM, como nos β-lactâmicos, ou C_{max}/CIM, como nos aminoglicosídeos, é insuficiente para avaliar de forma preditiva sua atividade clínica. Ao contrário, tem sido relatado que as determinações tanto da %T>CIM quanto da ASC_{24h}/CIM podem influenciar a atividade clínica dos macrolídeos considerados de geração avançada, como a azitromicina e a claritromicina (Amsden, 2001; Van Bambeke & Tulkens, 2001).

A investigação da influência dos parâmetros farmacocinéticos sobre o efeito dos antimicrobianos *in vivo* e sua aplicação na utilização racional de antimicrobianos têm sido amplamente discutidas, uma vez que a manutenção de concentrações séricas e

teciduais em função do tempo é essencial para atingir a eficácia terapêutica. Não há consenso quanto ao melhor método preditivo de atividade antimicrobiana dentre os modelos *in vitro* e *in vivo* reportados na literatura (Turnidge, 1998; Li, 2000; Amsden, 2001; Van Bambeke & Tulkens, 2001; Fridt-Moller, 2002; Toutain *et al*., 2002; Bonapace *et al*., 2002).

No presente trabalho, a eficácia *in vivo* da claritromicina na redução da microbiota oral e nasal de voluntários saudáveis foi investigada a partir da determinação dos parâmetros T>CIM, %T>CIM e $ASC_{0\text{-}24h}$/CIM, sendo descrita no Estudo 3.

2.3 Enzimas e transportadores intestinais como barreiras bioquímicas para a absorção de fármacos: modelos farmacocinéticos *in vitro*.

A biodisponibilidade de fármacos administrados por via oral pode ser afetada por diversos fatores físicos e biológicos, que incluem as propriedades de desintegração e dissolução da forma farmacêutica, a solubilidade da molécula no lúmen intestinal, sua permeabilidade na membrana plasmática e a susceptibilidade do fármaco a vários transportadores de membrana e enzimas de biotransformação intra ou extracelulares. A influência desses fatores sobre a biodisponibilidade sistêmica de um fármaco pode ser expressa pela equação:

$$F = F_{ab} * F_i * F_H = F_{ab} * (1 - E_i) * (1 - E_H)$$

onde F_{ab} representa a fração da droga absorvida através da membrana apical do enterócito, enquanto F_i e F_H representam as frações do fármaco que não sofrem efeito de primeira passagem intestinal e hepática, respectivamente (Hall *et al.*, 1999).

O advento da biologia molecular levou à identificação de inúmeras enzimas e transportadores no intestino humano. Embora o fígado seja considerado o principal local envolvido na biotransformação de fármacos, a presença de muitas enzimas metabolizadoras no intestino (tanto de fase I quanto de fase II) sugere uma importante participação deste órgão no metabolismo de primeira passagem de fármacos administrados por via oral. Tais enzimas são encontradas nas células epiteliais do intestino, os enterócitos e, embora apresentem concentrações muito inferiores às do fígado, possuem influência significativa

na biodisponibilidade de fármacos em função da grande superfície de contato entre a parede intestinal (Cummins *et al*., 2001).

O mais importante sistema de metabolismo oxidativo é o citocromo P450 (CYP), encontrado no retículo endoplasmático de hepatócitos e enterócitos, sendo responsável pela biotransformação de vários xenobióticos, hormônios endógenos e ácidos graxos, através de reações que incluem N-desmetilações, N-,S-oxidações, hidroxilações aromáticas e alifáticas (Cummins *et al*., 2001). Segundo Nelson *et al.* (1996), as enzimas do citocromo P450 são classificadas de acordo com sua relação evolutiva: CYP isoformas com 40% de identidade em aminoácidos são consideradas como de uma mesma família e classificadas de acordo com um algarismo arábico (ex. CYP1) e aquelas com identidade superior a 55% são agrupadas em sub-famílias designadas com letra maiúscula (ex. CYP1A). São conhecidas 18 famílias e 43 sub-famílias, das quais apenas as famílias 1, 2 e 3 parecem estar envolvidas na biotransformação de fármacos (Cummins *et al.*, 2001).

Embora um grande número de isoformas tenha sido caracterizado quanto à seletividade, regulação e função durante as décadas de 70 e 80, somente em 1987 a sub-família CYP3A foi descoberta no intestino de ratos e humanos através de estudos de indução por dexametasona (Watkins *et al.*, 1987), o que gerou futuras investigações sobre a função da CYP3A intestinal no metabolismo de primeira passagem (Cummins *et al.*, 2001).

A família CYP3A consiste de quatro sub-famílias: CYP3A4, a mais extensivamente estudada, CYP3A5, uma enzima polimórfica expressa no fígado de 25-30% da população, a CYP3A7, uma forma específica presente no fígado fetal e a CYP3A43, recentemente identificada e ainda pouco caracterizada funcionalmente (Domnaski *et al.*, 2001; Westlind *et al.*, 2001).

Dentre as isoformas do citocromo P450, a CYP3A4 é a mais abundante no fígado e no intestino, sendo estimada em 30% e 70% do total de CYP presente nestes órgãos, respectivamente (Shimada *et al.*, 1994). No fígado, a CYP3A4 encontra-se principalmente na zona 3 (centrilobular) dos hepatócitos. No intestino delgado, a CYP3A4 é altamente expressa tanto no duodeno como no jejuno, e em menor grau no íleo (Paine *et al.*, 1997). Localiza-se na camada de células epiteliais colunares do intestino, polarizada na região apical do enterócito (Kolars *et al.*, 1994).

Os enterócitos ainda apresentam proteínas transportadoras de captação (*uptake*) e efluxo (*efflux*) que podem facilitar ou dificultar a absorção de fármacos. A farmacocinética clássica considera a difusão passiva como sendo o principal sistema de permeação de fármacos através do epitélio intestinal. Recentemente, tem sido reconhecida a existência de proteínas capazes de mediar a captação de íons orgânicos (Tsuji & Tamai, 1999) através do transportador do ácido carboxílico (MCT, do inglês *monocarboxylic acid transporter*), bem como a captação de cátions orgânicos por transportadores específicos (OCTs, do inglês *organic cation transporters*), segundo Dresser *et al.* (2001). Além disso, alguns transportadores podem mediar tanto a captação de nutrientes quanto de fármacos; as cefalosporinas, por exemplo, são substratos para a captação facilitada H^+-dependente pelo peptídeo transportador PEPT1 (Inui & Terada, 1999).

O conhecimento destes sistemas tem sido utilizado no desenvolvimento de fármacos pouco absorvidos no intestino através da conjugação destes a aminoácidos e nucleosídeos, a fim de facilitar o reconhecimento por parte das proteínas transportadoras. Este processo aumentou de três a cinco vezes a área sob a curva do aciclovir, através da conjugação deste a um aminoácido éster, criando o valaciclovir (Oh *et al.*, 1999).

Por outro lado, transportadores de efluxo constituem uma barreira à absorção intestinal de fármacos. Dentre os transportadores já conhecidos, muitos pertencem à superfamília de transportadores ligados a ATP (*ATP-binding cassete transporter superfamily)*, que utilizam a energia proveniente do ATP para secretar fármacos contra um gradiente de concentração. A mais importante destas proteínas, identificada no intestino, é a glicoproteína P (P-gp do inglês *P-glycoprotein*), devido a sua ampla setividade e significativa expressão no intestino (Cummins *et al.*, 2001). A P-gp (formalmente classificada como ABCB1) foi descoberta nas células ovarianas de hamsters chineses, resistentes a colchicina (Juliano & Ling, 1976). Estas células também demonstraram resistência cruzada a uma série de fármacos quimioterápicos estruturalmente não relacionados, como a vinblastina, a daunomicina e a citocalasina B, mecanismo atribuído a uma proteína de superfície abundantemente expressa nessas células. Assim, denominou-se como glicoproteína P (P de permeabilidade) o produto da expressão do gene de

multiresistência a fármacos (MDR1, do inglês *multidrug-resistance gene*) (Juliano & Ling, 1976).

A descoberta da P-gp possibilitou o esclarecimento da múltipla resistência a quimioterápicos clinicamente observada em casos de câncer metastásico (Goldstein, 1996). Embora a P-gp tenha sido inicialmente observada em células cancerosas, foi constatada sua presença em tecidos humanos normais, como as células epiteliais do fígado, intestino, rins e pâncreas, na medula e córtex adrenais (Thiebaut *et al.*, 1987), células endoteliais do cérebro e na placenta (Cordon-Cardo *et al.*, 1989), e em células do sistema hematopoiético (Chaudahary *et al.*, 1992). A distribuição tecidual da P-gp sugere que esta proteína tem importante papel na eliminação de xenobióticos, bem como no controle da absorção e distribuição dos mesmos.

Wacher *et al.* (1995) relataram estreita similaridade entre os substratos metabolizados pelo CYP3A4 e conhecidos substratos ou inibidores da P-gp, bem como em sua distribuição tecidual, particularmente no fígado e intestino. Esta observação levou os autores a sugerirem a existência de uma interação entre estas proteínas, capaz de influenciar a absorção intestinal de fármacos e seu metabolismo.

Esta hipótese foi reforçada pela observação de que, no enterócito, a P-gp localiza-se na região apical da membrana citoplasmática, e a CYP3A4 situa-se no retículo endoplasmático; desta forma, a P-gp controlaria o acesso de fármacos ao metabolismo intracelular mediado pelo CYP3A4 (Wacher *et al.*, 1998).

Benet & Cummins (2001), revisaram os principais mecanismos pelos quais a atividade do citocromo P450, especificamente CYP3A4 e a P-gp poderiam interferir no transporte e metabolismo de fármacos, utilizando células Caco-2 modificadas para expressar estas estruturas. Segundo estes autores, o fármaco, geralmente hidrofóbico, é absorvido através de um processo passivo pelo enterócito, podendo ser metabolizado pelo CYP3A4, o principal sistema de biotransformação de fase I intestinal.

Entretanto, o fármaco também sofre co-transporte ativo pela P-gp, isto é, retorna ao lúmen intestinal e é reabsorvido, sendo exposto novamente à ação enzimática. Assim o papel da P-gp no intestino vai além da simples limitação da absorção do composto original, aumentando o acesso do fármaco ao metabolismo pelo CYP3A4 através de ciclos

repetidos de absorção e efluxo, o que pode acarretar na diminuição de sua biodisponibilidade. Ao contrário, os fármacos que não sofrem a ação da P-gp passam pelo enterócito apenas uma vez, não sendo observadas alterações na sua biodisponibilidade, a menos que a atividade da CYP3A4 seja induzida ou inibida através de agentes específicos (Benet & Cummins, 2001).

Uma linhagem de células de carcinoma de cólon humano, as células Caco-2, tem sido utilizada como modelo para a verificação do comportamento farmacocinético *in vitro* dos fármacos (Artursson & Borchardt, 1997). Estudos têm demonstrado que as taxas de permeação de fármacos através destas células correlacionam-se positivamente com a porcentagem de fármacos absorvidos no organismo tanto por difusão passiva quanto por transporte ativo (Artursson & Karlsson, 1991; Yee, 1997).

Transportadores de ácidos biliares, ácido monocarboxílico e dipeptídeos, bem como a P-gp, são expressos por células Caco-2 (Gutmann *et al.*, 1999). Entretanto, estas células não expressam o CYP3A4, a principal enzima oxidativa no intestino, limitando sua utilização no estudo das interações entre P-gp e CYP3A4. Tal inconveniente foi superado pela utilização de células Caco-2 modificadas para induzir a expressão de CYP3A4, através da adição de vitamina D3 ao meio de cultura (Schmiedlin-Ren *et al.*, 1997) ou de transferência de genes que expressam CYP3A4 (*CYP3A4-transfected Caco-2 cells* ou simplesmente CYP3A4-Caco-2), produzidas pelo laboratório Gentest (Gentest, Woburn, MA, USA).

A produção de proteínas como a P-gp e de NADPH redutase, o alto custo e a grande variabilidade no nível de indução de CYP3A4 limitam a utilização do modelo de células induzidas por vitamina D3 (Cummins *et al.*, 2001). Por outro lado, as células CYP3A4-Caco-2 atingem um nível de confluência em menor tempo, e a indução de CYP3A4 pode ser feita utilizando-se substâncias de menor custo. Este modelo tem sido utilizado para a investigação de diferentes fármacos, como o midazolam (Wacher *et al.*, 2001), o albendazol (Kobayashi *et al.*, 2001), a ciclosporina (Wacher *et al.*, 2001), compostos polifenólicos de atividade anticarcinogênica presentes no chá verde (Jodoin *et al.*, 2002), entre outros.

A claritromicina foi utilizada como objeto de estudo deste modelo, conforme descrito a seguir no Estudo 3. Acredita-se que a claritromicina, assim como a eritromicina, seja um substrato para a atividade do CYP3A4, para a P-gp e para um sistema de recaptação celular (Wacher *et al.*, 2001), o que poderia justificar sua baixa biodisponibilidade quando administrada por via oral (cerca de 55%).

A elucidação dos fatores que interferem no transporte e metabolismo da claritromicina pode contribuir para uma melhor compreensão de seu impacto na disposição deste fármaco no organismo e diferenças na biodisponibilidade de medicamentos similares, podendo ainda contribuir no ajuste de esquemas terapêuticos.

3 PROPOSIÇÃO

O objetivo geral deste trabalho foi o estudo de modelos farmacocinéticos *in vitro* e *in vivo*, através de ensaios cujos objetivos específicos foram:

- Determinar a biodisponibilidade comparativa da carbamazepina através de modelo de estudo de bioequivalência entre medicamentos teste e referência (estudo 1);

- Determinar a biodisponibilidade comparativa da claritromicina através de modelo de estudo de bioequivalência entre medicamentos teste e referência (estudo 2);

- Avaliar parâmetros de eficácia da claritromicina na redução da microbiota oral e nasal de voluntários saudáveis, correlacionando-os com os dados farmacocinéticos (estudo 3);

- Investigar os efeitos interativos de enzimas metabólicas e transportadores celulares na disposição da claritromicina (estudo 4).

4 MATERIAL E MÉTODOS

Esta tese está baseada na Informação CCPG/001/98/Unicamp que regulamenta o formato alternativo para tese de Doutorado e permite a inserção de artigos científicos de autoria ou co-autoria do candidato.

Assim sendo, esta tese é composta de quatro estudos que se encontram em fase de submissão ou já submetidos para publicação em revistas científicas, conforme descrito abaixo:

Estudo 1 – "Bioequivalence study of carbamazepine oral formulations available in Brazil." Submetido à Revista Brasileira de Ciências da Saúde.

Estudo 2 – "Comparative bioavailability of clarithromycin formulations in healthy Brazilian volunteers". Submetido ao Journal of Pharmaceutical Sciences.

Estudo 3 – "A PK/PD approach on the effects of clarithromycin on oral and nasal microbiota of healthy volunteers." Submetido ao Biological & Pharmaceutical Bulletin.

Estudo 4 – "The effects of the interactions between CYP3A4 and P-gp on the *in vitro* disposition of clarithromycin."

Os estudos 1 e 2 têm como princípio metodológico o modelo de estudos de biodisponibilidade e bioequivalência realizados segundo a Resolução RDC nº 84, a Resolução - RE nº 479, e a Resolução - RE nº 478, da ANVISA/MS, todas de 19 de março de 2002.

Os parâmetros farmacocinéticos utilizados no estudo 3 foram obtidos através de modelo de estudo de bioequivalência, conduzido de acordo com as diretrizes da ANVISA/MS, descritas acima. A eficácia *in vivo* da claritromicina na redução da microbiota oral e nasal de voluntários saudáveis foi investigada a partir da determinação dos parâmetros T>CIM, %T>CIM e $ASC_{0\text{-}24h}$/CIM, segundo modelos previamente reportados (Turnidge, 1998; Li, 2000; Amsden, 2001; Van Bambeke & Tulkens, 2001; Fridt-Moller, 2002; Toutain *et al*., 2002; Bonapace *et al*., 2002).

A investigação do comportamento farmacocinético *in vitro* da claritromicina em modelo de células CYP3A4-Caco-2 foi conduzida de acordo com o trabalho de Cummins e colaboradores (2001), tendo sido realizado durante o período de estágio no *Department of Biopharmaceutical Sciences*, *University of California, San Francisco* (UCSF, San Francisco, CA, USA) sob a supervisão do Prof. Dr. Leslie Z. Benet, com o apoio financeiro do programa PDEE/CAPES.

A seguir, serão apresentados material e métodos, resultados, discussão e conclusão de cada estudo, conforme submetidos à publicação.

5 RESULTADOS

5.1 Estudo 1

Bioequivalence Study of Carbamazepine Oral Formulations Available in Brazil.

Ana Paula Del Bortolo Ruenis[1]
Gilson Cesar Nobre Franco[2]
Sinvaldo Baglie[1,3]
Luciana Aparecida Tiberti[4]
Luiz Madaleno Franco[5]
Francisco Carlos Groppo[6]
Pedro Luiz Rosalen[6]

[1] Doutorando(a) em Farmacologia da FOP/UNICAMP e pesquisador(a) do Centro de Estudos de Bioequivalência – CEB da FOP/UNICAMP.

[2] Cirurgião-Dentista e estagiário do CEB da FOP/UNICAMP.

[3] Professor da UEPG-PR.

[4] Doutoranda e Química da Bioagri Laboratórios Ltda.

[5] Mestre em Farmacologia, Professor da UNIMEP e pesquisador do CEB da FOP/UNICAMP.

[6] Professor em Farmacologia e pesquisador do CEB da FOP/UNICAMP.

Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba
Piracicaba, SP, Brasil

State University of Campinas – UNICAMP
School of Dentistry of Piracicaba
Piracicaba, SP, Brazil

Correspondência/Correspondence:
Prof. Pedro Luiz Rosalen; Av. Limeira, 901; 13414-903 – Piracicaba, SP; Brazil
E-mail: rosalen@fop.unicamp.br

This study was supported by Laboratório Teuto-Brasileiro, GO, Brazil.

SUMMARY

The aim of this study was to compare the bioavailability of two carbamazepine tablet formulations produced in Brazil. The study was approved by local Ethical Committee and was performed using an open, randomized, two-period crossover design with 3 weeks interval between doses. A single 400mg oral dose of carbamazepine (Carmazin®, Laboratório Teuto-Brasileiro – test medication and Tegretol®, Novartis Biociências – reference medication) was administered to 24 healthy volunteers of both sexes (12 males and 12 females). The plasma concentrations of carbamazepine were quantified by a validated high-performance liquid chromatography (HPLC) method. The HPLC system used an UV-DAD detector, at 245nm. The data were submitted to variance analysis by ANOVA with 95% of Confidence Interval (CI). The geometric mean values (± sd) for AUC_{0-192} ($\mu g.h.mL^{-1}$), AUC_{0-inf} ($\mu g.h.mL^{-1}$), and C_{max} ($\mu g.mL^{-1}$), were 650.742 (± 176.657), 679.497 (± 205.340) and 10.897 (± 3.421) (test medication) and 673.479 (± 188.310), 695.254 (± 167.718) and 10.165 (± 2.165) (reference medication) respectively. CI for carbamazepine geometric mean of AUC_{0-t} and C_{max} ratios (test/reference) were: 0,84 – 1,11% and 0,91 – 1,25%. The corresponding geometric mean values of T_{max} (h) were 9.055 (± 13.458) (test) and 10.032 (± 9.570) (reference). The test medication was considered bioequivalent to the reference medication based on the rate and extent of absorption.

DESCRIPTORS: Bioequivalence, Carbamazepine, Bioavailability.

INTRODUCTION

Carbamazepine (CBZ) is one of the most common antiepileptic drugs, also used in the treatment of trigeminal neuralgia and psychiatric disorders, particularly bipolar depression (MASHFORD *et al*., 1974; SPINA *et al.*, 1996). Studies indicate that CBZ interact with voltage-gated sodium channels at therapeutic concentrations and reduce the frequency of sustained repetitive firing of action potentials in neurons (OKADA *et al.*, 2002).

CBZ is poorly soluble in aqueous media, and its gastrointestinal absorption is characterized as slow, erratic and possibly incomplete (JUNG *et al.*, 1997). Peak levels (Tmax) are usually achieved 4-12 hours after administration (BLAISE and BOURGEOIS, 2000). The drug is mainly metabolized by hepatic microsomal cytochrome P-450 isoenzymes to form carbamazepine-10, 11-epoxide (CBZ-E) and other derivatives. Carbamazepine 10, 11-epoxide seems to have antiepileptic properties as well as carbamazepine itself (LIU and DELGADO, 1994; BERNUS *et al.*, 1996; ROOYEN *et al.*, 2002).

Carbamazepine induces its own metabolism. As a result of this auto induction process, the half-life of CBZ decreases from 35.6 hours after a single dose to 20.9 hours after multiple doses; the average steady-state concentration of carbamazepine is reduced by 50% after 3 weeks of drug administration (SPINA *et al.*, 1996). Therapeutic use of CBZ has been associated with a number of side effects, such as neurological (diplopia, dizziness, tremor, headache, paraesthesias), cardiovascular (arrhythmia, hypertension or hypo tension), hepatic (increase of liver enzymes, hepatitis), hematological (anemia, leucopoenia, thrombocytopenia) and dermatological (exanthema, alopecia) effects (SCHIMIDT and BUHL, 1995).

Bioavailability studies of carbamazepine have been previously reported. SCHULTZ *et al.* (1992) evaluated the bioavailability of two commercial carbamazepine sustained-release formulations in 14 healthy male subjects in order to compare plasma concentration/time profiles and to determine the relative bioavailability of carbamazepine. This randomized study had a single-dose, crossover design and consisted of two trial

periods separated by a three-week washout period. A sensitive, validated HPLC method was used to analyze plasma carbamazepine levels. The 90% confidence limits for the AUC ratios were 109% (101-116%). These results indicated that the 2 medications of carbamazepine were bioequivalent.

REVANKAR *et al.* (1999) studied the bioavailability between two commercial carbamazepine sustained-release formulations. The results of C_{max}, T_{max}, $T_{1/2}$ and AUC for the conventional formulation (200 mg) were 2.43 (± 3.6) μg/mL, 6.5 (± 7.4) h, 44.6 (± 9.8) h and 178.8 (± 41.9) μg.h/mL.

The aim of this study was to compare the bioavailability of two carbamazepine tablet formulations produced in Brazil.

MATERIALS AND METHODS

Test and reference medications

The test medication (Carmazin® 400 mg of carbamazepine, lot n° 0383016, produced by Laboratório Teuto-Brasileiro) and the reference medication (Tegretol® 400 mg of carbamazepine, lot n° Z 80216, produced by Novartis Biociências) were supplied as tablets.

Clinical protocol

This study was performed on 24 healthy volunteers (12 males and 12 females), ranging in age from 18 to 35.5 years (23.85 ± 3.71 years), in weight from 44.5 to 85kg (69.52 ± 12.50kg) and in height from 1.53 to 1.84m (1.70 ± 0.07m). The subjects were informed of the purpose, protocol and risks of the study. Individuals with any prescription medications 14 days preceding and throughout the study, including over-the-counter products, or showing hypersensitivity to CBZ were excluded. The protocol was approved by a human subjects committee (FOP/UNICAMP, protocol n° 019/2002), and all subjects provided written informed consent.

The volunteers were free from significant cardiac, hepatic, renal, pulmonary, neurological, gastrointestinal and hematological disease as well as psychiatric disorders, as determined by their medical history, physical examination, ECG and routine laboratory tests (blood glucose, urea, creatinine, AST, ALT, alkaline phosphatase, γ-GT, total

bilirubin, albumin, total protein, triglyceride, total cholesterol, hemoglobin, hematocrit, total and differential white cell counts, erythrocyte sedimentation rate, and routine urinalysis). All subjects were negative for HIV, HBV and HCV. In female volunteers, βHCG was performed as pregnancy test (LERNER *et al.*, 2000).

The volunteers were hospitalized at 7:00 p.m. (night preceding the drug administration) and fasted 10 hours before dosing. Around 7:00 a.m., the subjects received a single oral dose of either Carmazin® as the test medication, or Tegretol® as the reference medication in an open, randomized, two-period crossover design with a minimum 3-week washout period between doses. Food was given 4, 8, 10, 12 and 24 hours after drug administration. No other food was permitted during the hospitalization period. Liquid consumption was permitted *ad libitium* 2 hours after administration, but xanthine-containing drinks including tea, coffee, and cola were avoided (KIM *et al.*, 2001).

Approximately 8 mL blood samples were collected via heparinized cannula at the following times: pre-dose, 3, 4, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 12, 24, 48, 72, 96, 120, 144, 168, 192 hours after the administration. The heparinized normal saline injection solution, 1 mL, was flushed after each blood sampling. Samples were collected in a centrifuge sterile tube containing 100μL of 10% EDTA solution. Every 2 hours, body temperature, systolic and diastolic arterial pressure and heart rate were recorded. The volunteers were instructed to inform any adverse event during all the study.

The blood samples were frozen at -20°C until the high-performance liquid chromatography (HPLC) analysis (JUNG *et al.*, 1997; CHELBERG *et al.*, 1988).

Bioanalytical assay

Materials

CBZ was from United States Pharmacopeia primary reference standards (USP – 100%), and the internal standard, 5,5 - Diphenylhytandoin (Phenytoin) was purchased from Sigma-Aldrich (99%). Methanol, acetonitrile and ethyl acetate were HPLC grade. Water used in the assay was obtained using a Millipore water purification system (Mili-Q System).

Apparatus

The HPLC system was an Alliance 2690 D Module – Waters, coupled to a 2487 Waters DAD detector (Waters, Milford, MA, USA), monitoring at 245 nm. Separations were performed using a RP-18 5 μm 4.6 x 250 mm analytical column (X-Terra, Waters, Milford, MA, USA). The mobile phase was water, acetonitrile and methanol (55:25:20) (v/v/v), with a flow rate of 1mL/min and column temperature at 35°C. A precolumn RP-18 5μm 3.0 x 20mm (X-Terra, Waters, Milford, MA, USA) was installed between the injector and the analytical column.

Standard solutions

CBZ work solution was prepared as 2.0 μg/mL solution in methanol. The standard solution of phenytoin was 150 μg/mL in methanol, and 100 μL of this solution was added to the plasma prior to extraction.

Sample preparation

The extraction procedure was performed by adding 100 μL of internal standard (IS) to a glass tube, followed by evaporation under nitrogen (N_2) at 40°C. Different volumes of standard solution, calculated according to the concentration of calibration or control standards, were added to fresh plasma to produce the calibration curve and the controls. After that, 500 μL of plasma was added to the tube and vortexed for 15 seconds. A 500 μL aliquot of 0.1M NaOH solution was added to the tube, vortexed during 15 seconds, followed by the addition of 4 mL ethyl acetate and vortexed for 1 minute. The resulting solution was centrifuged for 10 minutes at 3000 rpm and -2°C. Supernatant was separated, filtered with a 0.45 μm membrane filter and evaporated with nitrogen (N_2) at 40°C. The sample was then dissolved in 200 μL of mobile phase under ultrasonic bath at 40°C for 5 minutes, for further injection.

Method validation

Specificity assay was examined in relation to interferences from endogenous plasma constituents in the drug-free plasma of 8 healthy volunteers, plus lipemic and hemolised samples. The evaluation was made by comparing the chromatograms of blank

extracts with standard solutions of CBZ (100.0 μg/mL) and IS (30.0 μg/mL) prepared in methanol.

Calibration curves were developed using CBZ standard solutions in drug-free human plasma in concentrations of 0.75, 2.0, 5.0, 8.5, 12.0, 15.0, 18.0 and 20.0 μg/mL. The calibration curves were generated by plotting the ratio of the peak area of CBZ and internal standard against the CBZ concentration in solution, based on a simple linear model relating the CBZ concentration to the HPLC response. Linear regression techniques were used to assess the calibration curves with the determination of the correlation coefficient.

The limit of quantification (LOQ) was defined as the CBZ concentration at which the HPLC response was at least five times the response for the plasma blank and a concentration at which there was reproducible precision (% coefficient of variation of less than 20%) and accuracy (determined concentration being within 20% variation of the nominal concentration).

Quality control (QC) solutions were prepared at concentrations of 2.0, 8.5 and 15.0 μg/mL of CBZ in human plasma, representing, respectively, the low, mean and high concentration quality controls. The intra- and inter- day accuracy and precision were determined for each of these concentrations. Accuracy and precision were determined by using six replicate of fresh QC samples at each concentration on three separate days. Recoveries of CBZ and IS were determined by comparing the response of six extracted preparations at QC low, mean and high concentrations to the response of samples spiked with the authentic standard, after blank plasma extraction.

Analyte stability was assessed for all phases of the storage and analytical process by determining the accuracy and precision of the measured concentration compared to the nominal concentration. Freeze-thaw stability was evaluated for low (2.0 μg/mL), mean (8.5 μg/mL) and high (15.0 μg/mL) concentrations of CBZ in human plasma for three cycles by thawing at room temperature followed by refreezing to –20°C for 24h. Post-preparative (or autosampler) stability was determined using these same concentrations extracted and left at room temperature on the autosampler for 40h prior to injection on the HPLC system. Long-term stability study was performed using CBZ plasma samples at the same concentrations and stored at -20°C for 148 days.

Pharmacokinetics and statistical analysis

From the plasma concentration-time data, the following parameters for both carbamazepine formulations were obtained: C_{max}: the highest observed concentration during the 192 hours study period and T_{max}: the time at which C_{max} occurred (observed data), AUC $_{0\text{-}t}$: the area under the plasma concentration-time curve from baseline to time point (the last measurable or quantifiable time point for determination of $AUC_{0\text{-}\infty}$ and $AUC_{0\text{-}t}$ in this study was 192 hours after dosing for all subjects), $T_{1/2}$: elimination half-life, $AUC_{0\text{-}\infty}$: the area under the plasma concentration-time curve from baseline to time t (last time point with a measurable drug concentration), where was 192 hours for CBZ, calculated by the trapezoidal rule, plus the residual area, calculated as the concentration at time t divided by the elimination rate constant (McLEAN *et al.*, 2001). These parameters were determined using a software program (PK Solutions, Non-compartmental Pharmacokinetics Data Analysis Excel Template, 2001, Summit Research Services, Montrose, CO, USA).

The statistical analysis was performed using the SAS software (SAS 1999-2001, SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was performed on all variables (log transformed values) in order to estimate the residual error, which was used to construct the confidence interval, except for T_{max}. The effects considered in the ANOVA were: treatment, sequence, study period and subject within sequence. The 95% CI (confidence interval) of the geometric mean for the individual test/reference ratios for $AUC_{0\text{-}inf,}$ $AUC_{0\text{-}192}$ and C_{max} were obtained to assess the bioequivalence between the formulations. Data should be included in the acceptance range of 80-125%.

RESULTS

Twenty-four individuals completed the study, showing good health after clinical and laboratory assessment. During the hospitalization period, the reported adverse events were headache, nausea, sedation, diarrhea and muscular pain (Table 1).

Standard solutions of CBZ and phenytoin were injected to the HPLC system and were eluted at 7.7 and 8.6 min, respectively. Plasma obtained from six different individuals

was tested for interference and showed no interfering peaks at these retention times. The concentration-response relationship for the calibrations curve was described by regression analysis. The correlation coefficient of the calibration curve over the tested concentration range was 0.989 – 0.998, on each of the three days on which calibrations curves were run. The LOQ was consistently identified to be at 0.75 μg/mL using the defined criteria of acceptance.

The intra-day and inter-day precision values were within the limits for acceptance with coefficients of variation of less than 3.5% for the CBZ QC samples. Also, there is an acceptable level of accuracy across the range of QC sample concentrations tested for both intra-day and inter-day analysis (all within 85 to 115%). The mean percentage of recovery obtained for both analyte and IS were 93 and 73%, respectively.

The accuracy values of low, mean and high concentrations of CBZ in human plasma for 40 h room temperature stability (Table 2) and the three-cycle freeze-thaw stability (data not shown) were all acceptable. The accuracy of plasma samples after 148 days of storage at -20°C (Table 3) was higher than 93% for QC CBZ concentrations. The coefficient of variation for each stability study was lower than 15%.

Table 1

Table 2

Table 3

The mean plasma concentrations of carbamazepine (test and reference) obtained during the sampling period are presented in Figure 1.

Figure 1

Table 4 shows the mean values of pharmacokinetic parameters (AUC_{0-t}, AUC_{0-inf}, C_{max}, T_{max} and $T_{1/2}$) established from 24 healthy volunteers after the administration of

400mg of carbamazepine - Carmazin® (test medication) and Tegretol® (reference medication).

Table 4

The 95% CI for carbamazepine geometric mean $AUC_{0-t,}$ C_{max} and T_{max} ratios (test/reference) were: 0.84 – 1.11%, 0.91 – 1.25% and -6.60 – 6.94 h.

DISCUSSION

Carbamazepine formulations were well tolerated at the administered dose. The major side effect observed was headache that could be both related to the drug or to the long fasting period. After the second period of sampling, the volunteers were assessed as healthy based on physical examination and laboratory screening. Since all the subjects completed the study according to the protocol, the data obtained were eligible for pharmacokinetic evaluation.

The present study describes a new validated method for the CBZ analysis in plasma human samples. Similar results were reported by other authors; MINKOVA and GETOVA (2001) using a gas chromatographic system with mass spectrometric detection obtained a limit of detection of 10 μg/L and 92.5% recovery for CBZ. Although fast and sensitive, this method depends on more expensive equipments.

REVANKAR *et al.* (1999) studied the bioavailability between two commercial carbamazepine sustained-release formulations. The results of C_{max}, T_{max}, $T_{1/2}$ and AUC for the conventional formulation were 2.43 (± 3.6) μg/mL, 6.5 (± 7.4) h, 44.6 (± 9.8) h and 178.8 (± 41.9) μg/mL. YACOBI *et al.* (1999), in a 2-period, crossover 200 mg CBZ bioequivalence study found that pharmacokinetics parameters for both products at steady state were similar, with 90% and 95% confidence intervals falling within 90% and 110%.

According to FDA (Food and Drug Administration, USA), bioequivalent products means pharmaceutical equivalents or pharmaceutical alternatives whose rate (whose generally accepted measure is C_{max}) and extent of absorption (tested by comparing AUC)

do not show a significant difference when administered at the same molar dose under similar experimental conditions, either single or multiple dose (BENET, 1999). It is also stated that "two formulations that whose rate and extent of absorption differ by –20%/+25% or less are generally considered bioequivalent". This rule is only satisfied by statistical criteria, which show that the 90% confidence interval (CI) around the ratio of measured parameters will fall within the accepted 0.8 to 1.25 range, or 80 to 125% (BENET, 1999).

The importance of these criteria becomes clear analyzing the report by MEYER *et al.* (1992). The authors describe the bioavailability of three lots of a generic 200 mg CBZ tablet, which had been withdrawn from the market because of clinical failures reported after the use of these medications, compared to the bioavailability of one lot of the innovator product in healthy volunteers. The mean maximum carbamazepine plasma concentrations for two of the generic lots were only 61-74% that of the innovator product, while the third lot was 142% of the innovator. The mean AUC-time curve for the three generic lots ranged from 60 to 113% that of the innovator product. The results clearly indicate a significant difference in the rate and extent of absorption of the generic products compared to the innovator, as well as among the generic lots, showing that the generic product is bioinequivalent.

In conclusion, the results indicate that the two formulations of Carbamazepine (Carmazin® tablets 400 mg – test medication and Tegretol® tablets 400 mg – reference medication) are bioequivalent, and thus may be prescribed interchangeably.

REFERENCES

1. BENET LZ. Understanding bioequivalence testing. *Transplant Proc* 31(Suppl 3A):7S-9S, 1999.
2. BERNUS I, DICKINSON RG, HOOPER WD, EADIE MJ. Dose-dependent metabolism of carbamazepine in humans. *Epilepsy Res* 24:163-172, 1996.
3. BLAISE FD, BOURGEOIS MD. Pharmacokinetic properties of current antiepileptic drugs. *Neurology* 55(Suppl 3):S11-S16, 2000.

4. CHELBERG RD, GUNAWAN S, TREIMAN DM. Simultaneous high-performance Liquid Chromatographic determination of Carbamazepine and its principal metabolites in human plasma and urine. *Ther Drug Monit* 10(2):188-93, 1988.
5. JUNG H, MILÁN RC, GIRARD ME, LEÓN F, MONTOYA MA. Bioequivalence study of carbamazepine tablets: in vitro/in vivo correlation. *Int J Pharm* 152:37-44, 1997.
6. KIM YG, KIM HJ, KWON JW, LEE MG. Bioequivalence of clarithroomycin tablet formulations assessed in Korean males. *Int J Pharmacol Ther* 39(8):356-361, 2001.
7. LERNER FE, CALIENDO G, SANTAGADA V, SANTANA GSM, MORAES MEA, DE NUCCI G. Clarithromycin bioequivalence study of two oral formulations in healthy human volunteers. *Int J PharmacolTher* 38(7):345-354, 2000.
8. LIU H, DELGADO MR. Influence of Sex, Age, Weight, and Carbamazepine Dose on Serum Concentration Ratios, and Level/Dose Ratios of Carbamazepine and Its Metabolites. *Ther Drug Monit* 16:469-476, 1994.
9. MASHFORD ML, RYAN PL, THOMSON WA. Determination of Carbamazepine in plasma. *J Chromatogr* 89:11-15, 1974.
10. McLEAN A, BROWNE S, ZHANG Y, SLAUGHTER E, HALSTENSON C, COUCH R. The influence of food on the bioavailability of a twice-daily controlled release carbazepine formulation. *J Clin Pharmacol* 41:183-186, 2001.
11. McLEAN A, BROWNE S, ZHANG Y, SLAUGHTER E, HALSTENSON C, COUCH R. The influence of food on the bioavailability of a twice-daily controlled release carbazepine formulation. *J Clin Pharmacol* 41:183-186, 2001.
12. MEYER MC, STRAUGHN AB, JARVI EJ, WOOD GC, PELSOR FR, SHAH VP. The bioinequivalence of carbamazepine tablets with a history of clinical failures. *Pharm Res* 9(12):1612-6, 1992.
13. MINKOVA G, GETOVA D. Determination of carbamazepine and its metabolite carbamazepine-10,11-epoxide in serum with gas-chromatography mass spectrometry. *Methods Find Exp Clin Pharmacol* 23(9):481-5, 2001.

14. KADA M, ZHU G, YOSHIDA S, KANAI K, HIROSE S, KANEKO S. Exocytosis mechanism as a new targeting site for mechanisms of action of antiepileptic drugs. *Life Sci* 72:465-473, 2002.
15. REVANKAR SN, BHATT AD, DESAI ND, BOLAR HV, SANE SP, TIPNIS HP. Comparative bioavailability study of a conventional and two controlled release oral formulations of Tegretol (carbamazepine) – 200 mg. *J Assoc Physicians India* 47:886-889, 1999.
16. ROOYEN GF, BADENHORST D, SWART KJ, HUNDT HKL, SCANES T, HUNDT AF. Determination of carbamazepine and carbamazepine 10, 11-epoxide in human plasma by tandem liquid chromatography-mass spectrometry with electrospray ionization. *J Chromatogr B* 769:1-7, 2002.
17. SCHIMIDT S, BUHL MS. Signs and symptoms of carbamazepine overdose. *J Neuro* 242:169-173, 1995.
18. SCHULZ HU, DUSING R, LUHRMANN B, FRERCKS HJ. Investigation of the bioequivalence of two sustained-release formulations in healthy subjects. *Int J Clin Pharmaco*l 30:410-414, 1992.
19. SPINA E, PISANI F, PERUCCA E. Clinically Significant Pharmacokinetic Drug Interactions with Carbamazepine. *Clin Pharmacokinet* 31(3):198-214, 1996.

Table 1. Number of volunteers that reported adverse events after a single oral dose of carbamazepine 400mg.

Effect	**Carmazin® 400mg (Test)**	**Tegretol® 400mg (Reference)**
Headache	5	6
Nausea	1	-
Sedation	1	2
Diarrhea	-	1
Muscular pain	1	1

Table 2. Values of low, mean and high concentrations of carbamazepine (CBZ) in auto sampler stability assay.

	T = 0 h			T = 40 h		
	QC low	QC mean	QC high	QC low	QC mean	QC high
CBZ (µg/mL)	1.999	8.659	15.472	1.988	8.648	15.177
SD	0.072	0.104	0.856	0.092	0.097	0.913
CV (%)	3.58	1.20	5.53	4.62	1.12	6.02
Accuracy	99.93	101.87	103.15	99.42	101.74	101.18
Stability (%)*				-0.55	-0.13	-1.91

*Stability (%) = (CBZ concentration at T = 40 h - CBZ concentration at T = 0 h) x 100

CBZ concentration at T = 0 h

Table 3. Values of low, mean and high concentrations of carbamazepine (CBZ) obtained after 148 days of storage at – 20°C.

	T = 0 days (fresh samples)			T = 148 days (frozen samples)		
	QC low	QC mean	QC high	QC low	QC mean	QC high
CBZ (μg/mL)	2.029	8.612	15.736	1.885	8.314	14.041
SD	0.059	0.198	1.097	0.018	0.044	0.469
CV (%)	2.91	2.30	6.97	0.97	0.53	3.34
Accuracy	101.47	101.32	104.91	94.25	97.81	93.61
Stability (%)*				-7.10	-3.46	-10.77

*Stability (%) = (CBZ concentration at T = 148 days - CBZ concentration at T = 0 days) x 100
CBZ concentration at T = 0 days

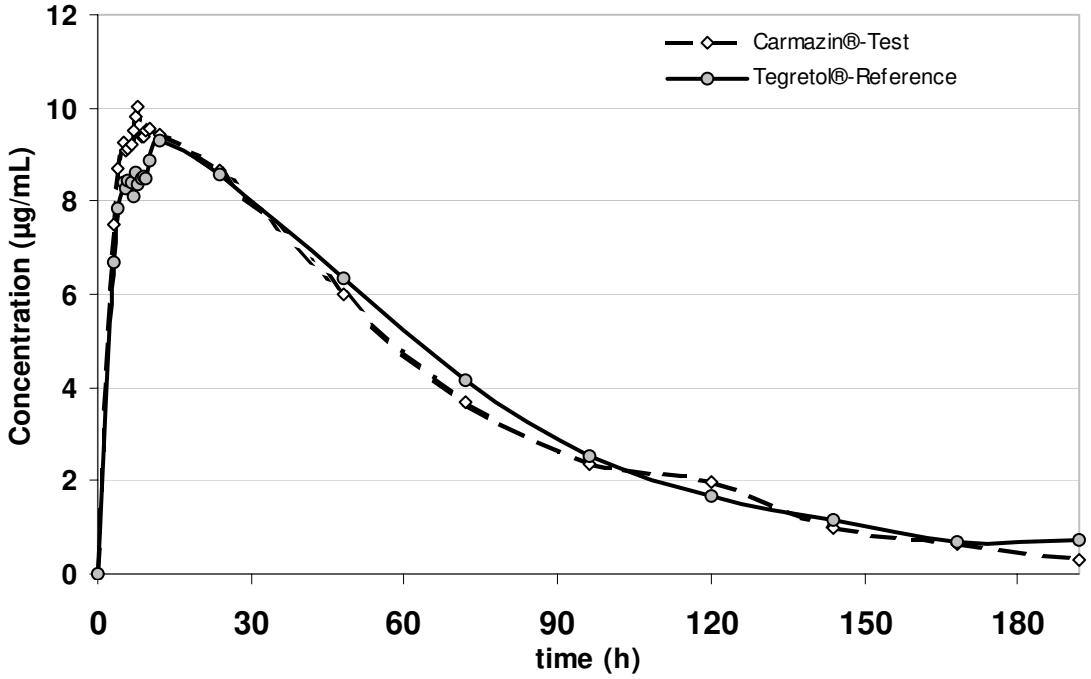


Figure 1. Concentration versus time curves. Mean plasma concentrations (µg/mL) of carbamazepine (test and reference) obtained from 24 volunteers.

Table 4. Geometric mean (± sd) values of the pharmacokinetic parameters for 24 volunteers after the administration of carbamazepine formulations.

Pharmacokinetic Parameter	**Carmazin® 400mg (Test)**	**Tegretol® 400mg (Reference)**
AUC_{0-192} ($\mu g.h.mL^{-1}$)	650.742 (± 176.657)	673.479 (± 188.310)
AUC_{0-inf} ($\mu g.h.mL^{-1}$)	679.497 (± 205.340)	695.254 (± 167.718)
C_{max} ($\mu g.mL^{-1}$)	10.897 (± 3.421)	10.165 (± 2.165)
T_{max} (h)	9.055 (± 13.458)	10.032 (± 9.570)
$T_{1/2}$ (h)	52.586 (± 52.711)	53.459 (± 31.544)

5.2 Estudo 2

Comparative bioavailability of clarithromycin formulations in healthy Brazilian volunteers.

Ana Paula Del Bortolo Ruenis, PharmD, MS, CCRP[1]
Ronilson A. Moreno, PhD[2]
Eduardo Abib Júnior, M.D. M. Sc[2].
Roberta Pessoa Simões, PharmD, PhD[3]
Luiz Madaleno Franco, PharmD, MS[4]
Francisco Carlos Groppo, DDs, PhD[5]
Pedro Luiz Rosalen, PharmD, PhD[5]

[1] Certified Clinical Research Professional (SoCRA), Ph.D student, Area of Pharmacology, Anesthesiology and Therapeutics - FOP/UNICAMP, Reseacher of the Bioequivalence Studies Unit - FOP/UNICAMP.

[2] Reseacher of Synchrophar Assessoria e Desenvolvimento de Projetos Clínicos.

[3] Reseacher of the Bioequivalence Studies Unit - FOP/UNICAMP.

[4] Professor of UNIMEP and Reseacher of the Bioequivalence Studies Unit - FOP/UNICAMP

[5] Professor of Pharmacology and Reseacher of the Bioequivalence Studies Unit - FOP/UNICAMP.

Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba
Piracicaba, SP, Brasil

State University of Campinas – UNICAMP
School of Dentistry of Piracicaba
Piracicaba, SP, Brazil

Correspondence:
Prof. Pedro Luiz Rosalen; Av. Limeira, 901; 13414-903 – Piracicaba, SP; Brazil
E-mail: rosalen@fop.unicamp.br

Abstract

The objective of this study was to assay the comparative bioavailability of clarithromycin in healthy volunteers. The study was conducted using an open, randomized, two-period crossover design with 1-week interval between doses. A single 500 mg oral dose of each formulation was administered to 24 healthy volunteers of both sexes (12 males and 12 females). Blood samples were collected at pre-dose, 0.33, 0.66, 1, 1.33, 1.66, 2, 2.5, 3, 4, 6, 8, 10, 12, 16, 20 and 24 h after the administration. Clarithromycin plasmatic concentrations were quantified by a validated LC-MS-MS method. The full-scan single-mass spectrum and the daughter ion-mass spectrum for CLR and IS were (m/z) 748.3 > 157.7 and 837.5 > 157.6, respectively. The retention times for both compounds were 4.0 minutes. The limit of quantification (LOQ) was 0.02 μg/mL. The data were submitted to ANOVA with 90% of Confidence Interval (CI). Intra-individual CV% values were 14.25% and 12.62%, respectively for C_{max} and AUC_{0-24}. The geometric mean values (± sd) for AUC_{0-24} (μg.h/mL), AUC_{0-inf} (μg.h/mL), and C_{max} (μg/mL) for test medication were 18.56 (± 6.87), 18.8 (± 5.70) and 2.45 (± 0.88); the obtained values for reference medication were 18.29 (± 5.39), 19.10 (± 7.21) and 2.5 (± 0.69). 90% CI for clarithromycin geometric mean of AUC_{0-24}, AUC_{0-inf} and C_{max} ratios (test/reference) were: 93.6 – 105.9%, 93.8 – 106.2% and 89 – 103.2%. The test medication was considered bioequivalent to the reference medication based on the rate and extent of absorption.

DESCRIPTORS: Bioequivalence, clarithromycin, LC-MS-MS.

Introduction

Clarithromycin (CLR) is a large spectrum macrolide antimicrobial agent, similar to erythromycin against gram-positive and gram-negative microorganisms, atypical pathogens and some anaerobes.[1)] Its activity is enhanced by the *in vivo* formation of its active metabolite 14-hidroxiclarithromycin (14-OH), showing synergistic activity against various pathogens including *Haemophyllus influenzae, Moraxella catarrhalis, Legionella* sp. and also streptococci and staphylococci.[2)]

Macrolide antibiotics, including clarithromycin and its active metabolite, are bacteriostatic agents that inhibit protein synthesis by binding reversibly to 50S ribosomal sub-units of sensitive microorganisms.[3, 4)]

Oral daily recommended dosage to adult patients is usually 250 to 500 mg every 12 hours, during 7 to 14 days, and 7.5 mg/kg twice daily (up to 250 mg/day) during 5 to 10 days for pediatric patients, depending on the infection severity and etiological agent. Infections caused by *Mycobacterium avium* complex or toxoplasmosis encephalitis in AIDS patients must be treated with 1 to 2 g/daily.[5)]

Pharmacokinetic parameters are an important tool that might be observed in dosage regimens. Previously observed mean C_{max} values ranging from 1.77 to 1.89 μg/mL after a single 500 mg clarithromycin dose; T_{max} was achieved in 3 hours. C_{max} and T_{max} values obtained for 14-OH were 0.8 μg/mL and 3 h, respectively.[1)] Protein binding of clarithromycin has been shown to range 40 to 70 %, and is dose dependent.[6)] Clarithromycin oral bioavailability is 55%, because of its rapid first-pass metabolism.[7)]

The parent compound shows non-linear elimination kinetics, suggesting that elimination is saturable. AUC values increased from 4.1 μg.h/mL (250 mg dose) to 53 μg.h/mL after a 1200 mg dose. Significant increase in $T_{1/2\beta}$, from 4.39 h to 11.27 h and clearance decrease observed in this study supports this hypothesis.[8)]

Measurement of clarithromycin and its active metabolite 14-OH in body fluids can be performed using high performance liquid chromatography (HPLC) techniques with electrochemical detection, as previously described.[9)] Reversed phase liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) was used to quantify the parent

compound and its active metabolite. This method requires a simple extraction procedure, also reducing the analysis time.[10)]

The aim of this study was to compare the bioavailability of two clarithromycin tablet medications (test and reference) in 24 healthy Brazilian volunteers, using a validated LC-MS-MS method.

Materials and methods

Test and reference medications

The test medication (Claritromicina Merck® 500 mg of clarithromycin, produced by Merck S.A Indústrias Químicas) and the reference medication (Klaricid® 500 mg of clarithromycin, produced by Abbott Laboratórios do Brasil, Ltda) were supplied as tablets.

Clinical protocol

This study was conducted according to the I.C.H Harmonized Tripartite Guideline for Good Clinical Practice (1996), the Declaration of Helsinki (1965) and Resolutions 196 (1996) and 251 (1997) – National Health Council, Health Ministry, Brazil. This study enrolled 24 healthy volunteers (12 males and 12 females), ranging in age from 18 to 45 years, in weight from 50 to 90 kg (64.42 ± 9.53 kg) and in height from 1.50 to 1.80 m (1.64 ± 0.08 m). The subjects were informed of the purpose, protocol and risks of the study. Individuals with any prescription medications 14 days preceding the study and throughout the study, including over-the-counter products and hypersensitivity to clarithromycin were excluded. The protocol was approved by a human subjects committee (FCM/UNICAMP, protocol # 190/2001), and all subjects provided written informed consent.

The volunteers were free from significant cardiac, hepatic, renal, pulmonary, neurological, gastrointestinal and hematological disease as well as psychiatric disorders, as determined by their medical history, physical examination, ECG and routine laboratory tests (blood glucose, urea, creatinine, AST, ALT, alkaline phosphatase, γ-GT, total bilirubin, albumin, total protein, triglyceride, total cholesterol, hemoglobin, hematocrit,

total and differential white cell counts, erythrocyte sedimentation rate, and routine urinalysis). All subjects were negative for HIV, HBV and HCV. In female volunteers, βHCG was performed as pregnancy test.

The volunteers were hospitalized at 8:00 p.m. (night preceding the drug administration) and fasted 10 hours before dosing. At 7:00 a.m., the subjects received a single oral dose of either Clarithromycin Merck® as the test formulation, or Klaricid® as the reference formulation in an open, randomized, two-period crossover design with a minimum 1-week wash-out period between doses. Food was given 4, 8, 10, 12 and 24 hours after drug administration. An evening meal was provided 8 h after dosing. No other food was permitted during the hospitalization period. Liquid consumption was permitted *ad libitium* 2 hours after administration, but xanthine-containing drinks including tea, coffee, and cola were avoided.

Approximately 8 mL blood samples were collected via heparinized cannula at the following times: pre-dose, 0.33, 0.66, 1, 1.33, 1.66, 2, 2.5, 3, 4, 6, 8, 10, 12, 16, 20 and 24 h after the administration. The heparinized normal saline injection solution, 1 mL, was flushed after each blood sampling. Samples were collected in a centrifuge sterile tube containing heparin solution. Every 2 hours, body temperature, systolic and diastolic arterial pressure and heart rate were recorded. The volunteers were instructed to inform any adverse event during all the study.

The blood samples were centrifuged at 4550 X *g* for 5 minutes (4°C) and plasma was stored at -20°C for further LC-MS-MS analysis.

Bioanalytical assay

Materials

CLR was provided by a pharmaceutical company (Medley Lab, Brazil), and the internal standard, roxithromycin (IS) was purchased from Sigma-Aldrich. Methanol, acetonitrile, formic acid and ammonium acetate were HPLC grade. Water used in the assay was obtained using a Millipore water purification system (Mili-Q System).

A Hewlett-Packard HPLC system (G1312-UM) coupled to a Micromass Quattro II triple stage quadrupole mass spectrometer, equipped with API electrospray source was

used. Separations were performed using a C18 Genesis 4 μm 150 mm x 4.6 mm i.d. analytical column at 40°C. The mobile phase was 57% acetonitrile, 25% water and 18% methanol, added by formic acid 10 mM (water) and ammonium acetate 20 mM. The flow rate was 0.7 mL/min and the total run time was 5.5 minutes. The mass spectrometer was run in the positive mode (ES+) and set for multiple reaction monitoring (MRN). The full-scan single-mass spectrum and the daughter ion-mass spectrum for CLR and IS were (m/z) 748.3 > 157.7 and 837.5 >157.6, respectively. The retention times for both compounds were 4.0 minutes.

Sample preparation

Both CLR and IS work solutions were prepared as 1.0 mg/mL solution in methanol/water (50:50 v/v). The extraction procedure was performed by transferring 200 μL of plasma to test tubes, followed by the addition of 50 μL of IS work solution. The samples were vortexed for 10 seconds and 4 mL of diethyl ether and dichloromethane (70:30 v/v) were added to each tube. After vortexing during 40 seconds, the organic phase was removed to clean tubes. The solvent was evaporated under nitrogen (N_2) at 37^{o}C. Each sample was then diluted in 200 μL of mobile phase and transferred to HPLC vials, for further injection.

Method validation

Specificity assay was examined in relation to interferences from endogenous plasma constituents in the drug-free blank plasma samples of six volunteers (normal, hyperlipemic and haemolized), by comparing these chromatograms of blank plasma samples to those obtained with aqueous solutions of CLR (0.02 and 10.0 μg/mL) and IS (10.0 μg/mL).

Calibration curves were developed by adding CLR standard solutions in drug-free human plasma in order to achieve concentrations of 0.02, 0.03, 0.05, 0.10, 0.20, 0.50, 1.0, 2.0, 5.0 and 10.0 μg/mL. The limit of quantification (LOQ) was defined as the CLR concentration at which the HPLC response was at least five times the response for the plasma blank and a concentration at which there was reproducible precision (% coefficient of variation of less than 20%) and accuracy (determined concentration being within 20% variation of the nominal concentration).

Quality control (QC) solutions were prepared at concentrations of 0.05, 0.5, 2.0 and 8.0 μg/mL of CLR in human plasma, representing, respectively, the low (A), mean (0.5 and 2.0 μg/mL, B and C) and high (D) concentration quality controls. The intra-day accuracy (experimental/nominal concentrations ratio, expressed as percentage) and precision (CV %) were determined for each of these concentrations (8 replicates). Inter-day accuracy and precision were determined by using eight replicates of fresh QC samples at each concentration, in three separate days.

CLR recoveries (n=5) and IS (n=10) were evaluated by calculating the mean area or response of low (0.05 μg/mL), mean (0.5 μg/mL) and high (5.0 μg/mL) QC concentrations (IS concentration = 1.25 μg/mL) and dividing the extracted sample mean by the unextracted sample mean of the corresponding concentration, expressed as percentage.

Analyte stability was assessed for all phases of the storage and analytical process by determining the accuracy and precision of the measured concentration compared to the nominal concentration. Freeze-thaw stability was evaluated for low (0.05 μg/mL), mean (0.5 μg/mL) and high (5.0 μg/mL) concentrations of CLR (IS concentration = 1.25 μg/mL) in human plasma for three cycles by thawing at room temperature followed by refreezing to –20°C for 24h. Post-preparative stability was determined using these same concentrations extracted and left at room temperature for 6 h and at the auto sampler (5°C) for 48 h prior to injection on the HPLC system. Long-term stability study was performed using CLR and IS plasma samples at the same concentrations and stored at -20°C for 13 days.

Pharmacokinetic and statistical analysis

From the plasma concentration-time data, the following parameters for both clarithromycin formulations were obtained: C_{max}: the highest observed concentration during the 24 hours study period and T_{max}: the time at which C_{max} occurred, AUC_{0-t}: the area under the plasma concentration-time curve from baseline to time point (the last measurable or quantifiable time point for determination of $AUC_{0-\infty}$ and AUC_{0-t} in this study was 24 hours after dosing for all subjects), $T_{1/2}$: elimination half-life, $AUC_{0-\infty}$: the area under the plasma concentration-time curve from baseline to time t (last time point with a measurable drug

concentration), that was 24 hours for clarithromycin calculated by the trapezoidal rule, plus the residual area, calculated as the concentration at time t divided by the elimination rate constant. These parameters were determined by software programs (WinNonlin Professional Network edition, version 1.5m; Bioequivalence Program for Two-Period Cross-Over Studies, version 3.4).

The statistical analysis was performed using the SAS software (SAS 1999-2001, SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was performed on all variables (log transformed values) in order to estimate the residual error, which was used to construct the confidence interval, except for T_{max}. The effects considered in the ANOVA were: treatment, sequence, study period and subject within sequence. The 90% CI (confidence interval) of the geometric mean for the individual test/reference ratios for $AUC_{0-\infty}$, AUC_{0-24} and C_{max} were obtained to assess the bioequivalence between formulations. Data should be included in the acceptance range of 80-125%.

Results

There were no interfering peaks at the retention times of CLR and IS or they were less than 20% or 5% of the response of the LOQ, respectively for CLR and IS. The linearity of the method was demonstrated by the calibration curve (r^2= 0.994260), with a LOQ of 0.02 μg/mL (CV= 6.5%). CLR recoveries for low, mean and high concentrations were respectively 92.7%, 85.2% and 93.6%, while 75.2% of IS was recovered after extraction. Intra-day and inter-day accuracy and precision results were within the acceptance range (20% for LOQ and 15% for other QCs), as summarized at Table 1.

Table 1. Intra- and inter-day mean QC values, precision and accuracy for the assay of clarithromycin.

Sample	Intra-day assay			Inter-day assay		
Spiked (μg/mL)	**Mean (μg/mL)**	**Precision CV%**	**Accuracy (%)**	**Mean (μg/mL)**	**Precision CV%**	**Accuracy (%)**
LOQ (0.02)	**0.018**	**4.5**	**90**	**0.02**	**9.3**	**100**
QCA (0.05)	**0.047**	**4.6**	**94**	**0.05**	**9.1**	**100**
QCB (0.5)	**0.52**	**4.2**	**104**	**0.53**	**4.8**	**106**
QCC (2.0)	**2.2**	**1.3**	**110**	**2.2**	**3.2**	**110**
QCD (8.0)	**8.5**	**4.2**	**106.2**	**8.6**	**4.0**	**107**

Auto sampler, freeze and thaw, short-term and long-term stability tests demonstrated that neither CLR nor IS showed significant degradation (Student's t-test, $p>0.05$), in the described conditions.

Clarithromycin formulations were well tolerated at the administered dose. After the second period of sampling, the volunteers were assessed as healthy based on physical examination and laboratory screening. Since all the subjects completed the study according to the protocol, the data obtained were eligible for pharmacokinetics evaluation. The mean plasmatic CLR concentrations for 24 volunteers are presented in Figure 1.

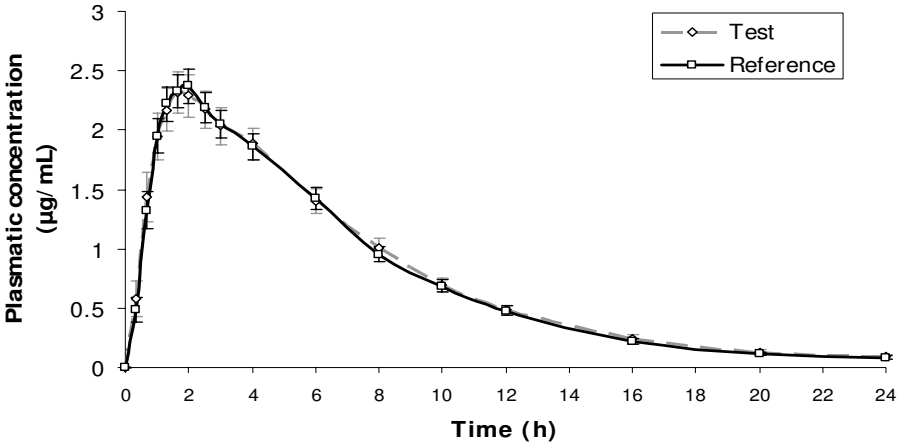


Figure 1. Concentration versus time curves. Mean plasmatic concentrations and S.D. of clarithromycin obtained from 24 volunteers, regarding test and reference medications.

The pharmacokinetics parameters obtained in this study are summarized at Table 2. The inter- and intra-individual CVs% for the absorption parameters (AUC_{0-24} and C_{max}) were assessed by ANOVA, according to ANVISA requirements. Inter-individual difference considering volunteer within sequence was not observed. Intra-individual CV% values were 14.25% and 12.62%, respectively for C_{max} and AUC_{0-24}.

The geometric mean values, standard deviation and the coefficient of variation (%) for AUC_{0-24}, $AUC_{0-\infty}$, C_{max}, T_{max}, $T_{1/2}$, Vd/F, CL/F and K_{el} are shown in Table 2.

Table 2. Geometric mean, ± SD and CV% values of the pharmacokinetic parameters for 24 volunteers after the administration of clarithromycin test and reference formulations.

	Claritromicina Merck® 500 mg (test)			**Klaricid® 500 mg (reference)**		
PK parameter	**Mean**	**± SD**	**CV (%)**	**Mean**	**± SD**	**CV (%)**
AUC_{0-24} ($\mu g.h.mL^{-1}$)	**18.56**	**6.87**	**37.01**	**18.29**	**5.39**	**29.46**
$AUC_{0-\infty}$ ($\mu g.h.mL^{-1}$)	**18.80**	**5.70**	**30.31**	**19.10**	**7.21**	**37.74**
C_{max} ($\mu g.mL^{-1}$)	**2.45**	**0.88**	**35.91**	**2.50**	**0.69**	**27.60**
T_{max} (h)	**1.80**	**0.52**	**28.88**	**1.83**	**0.50**	**27.32**
K_{el} (h^{-1})	**0.16**	**0.03**	**18.75**	**0.17**	**0.04**	**23.53**
$T_{1/2}$ (h)	**4.52**	**0.95**	**21.01**	**4.37**	**1.15**	**26.31**
Vd/F (l/kg)	**3.25**	**0.68**	**18.46**	**3.13**	**0.81**	**25.88**
Cl/F (ml/min/kg)	**29.53**	**10.03**	**33.96**	**29.11**	**9.43**	**32.39**

The 90% CI for clarithromycin geometric mean AUC_{0-24}, $AUC_{0-\infty}$ and C_{max} ratios (test/reference) were: 93.6-105.9%, 93.8-106.2% and 89.0-103.2%.

Discussion

CLR plasma concentrations have been determined by different methods. A described HPLC method with electrochemical detection to quantify CLR plasmatic concentrations was used in many studies.[7,11,12,13,14)] This method showed linearity ($r^2 \geq 0.99$), precision (within-day and day-to-day with CV% mostly within ± 5%), accuracy routinely within 90-110% of theoretical values and recoveries of ≥ 85%. However, this method achieved LOQs within the range of 0-2 μg/mL, with limits of quantification of approximately 10.03 μg/mL. A HPLC method with amperometric detection showed linearity of the calibration curves over the concentration ranges 0.03-3.0 μg/mL for CLR, but this method was validated for rat plasma samples.[15)] A HPLC method for the determination of the CLR using a fluorescence detector that showed linearity, accuracy and precision, but high LOQ (0.19 μg/mL) and total run time of 15.7 minutes.[16)]

The LC-MS-MS analysis previously described obtained a LOQ of 5 ng/ml, within- and between-run plasma QC coefficients of variation of 5.8% and 15.7%, and inaccuracy within- and between-runs of 14% and 17%, respectively.[10)] In the present study, this method was modified and validated, improving the sensitivity, accuracy and precision of the analytical assay, as demonstrated at Table 1.

Pharmacokinetic parameters found in previous studies support data obtained in the present trial, since similar results are reported in literature. After a single 500 mg oral dose of CLR, C_{max} was achieved in 2 hours, showing mean values of 1.77 to 1.89 mg/L and AUC values of 11.06 to 11.66 μg.h/mL, quantified by HPLC.[12)] It was also demonstrated that CLR concentrations after a single oral dose of 500 mg were 2.72 ± 0.87, 1.21 ± 0.34 and 0.73 ± 0.35 mg/l after 2, 7 and 12 hours respectively.[17)] Another study showed a C_{max} of 2.98 mg/L in plasma obtained from human healthy volunteers after a 500 mg dose of clarithromycin b.i.d, in the first day.[18)] C_{max} and AUC values ranging from 2.73 μg/mL to 2.95 μg/mL and 19.74 μg.h/mL to 20.78 μg.h/mL were also reported.[10)] Similar results were obtained in the present study (C_{max} and AUC ranging from 2.45 to 2.50 mg/L and 18.56 to 18.29 μg.h/mL, for reference and test medications, respectively).

CLR inter- and intraindividual variabilities for absorption parameters have not been described in scientific literature. It has been reported that the major source of pharmacokinetic intraindividual variability appears to be the presence of significant gut counter transport, mediated by P-glycoprotein (P-gp), and metabolism due to intestinal cytochrome P-450 3A4 (CYP3A4) for intermediate and low hepatic extraction drugs. Variabilities observed in high hepatic extraction drugs would be explained by the presence of significant hepatic first pass loss.[19] Drugs which exhibit high intraindividual variabilities (CVs >30%) are major substrates for P-gp and CYP3A4, and may suffer a decrease in their bioavailabilities.[19] In this study, CLR showed intraindividual variabilities of 14.25% and 12.62%, respectively for C_{max} and AUC_{0-24}. The number of subjects enrolled in the study was enough to reach the 90% CI values within the acceptance range.

According to FDA (Food and Drug Administration, USA), bioequivalent products means pharmaceutical equivalents or pharmaceutical alternatives whose rate (whose generally accepted measure is C_{max}) and extent of absorption (tested by comparing AUC) do not show a significant difference when administered at the same molar dose of the therapeutic moiety under similar experimental conditions, either single or multiple dose.[20,21] This rule is only satisfied by statistical criteria, which might show that the 90% CI values around the ratio of measured parameters fall within the accepted 0.8 to 1.25 range, or 80 to 125%.[21] Data obtained in this study clearly indicate that both test and reference 500 mg clarithromycin formulations are bioequivalent, and thus may be prescribed interchangeably.

Acknowledgment

This study was supported by a grant from Merck S.A Indústrias Químicas. The author Ana Paula Del Bortolo Ruenis was supported by a scholarship provided by CNPq.

References

1. Fraschini, F., *J. Int. Med. Res.*, **18**, 171-176 (1990)

2. Jones, R.N., Erwin, M.E., Barrett, M.S., *Eur. J. Clin. Microbiol. Infect. Dis.*, **9**, 846-848 (1990).
3. Kakegawa, T., Ogawa, T., Hirose, S., *Chemotherapy*, **36**, 123-128 (1988)
4. Kakegawa, T., Hirose, S., *Chemotherapy*, **38**, 317-323 (1990)
5. Montgomery, E.H. *In*: NEIDLE, E.A., YAGIELA, J.A. *Farmacologia e terapêutica para dentistas.,* 397-411 (1991)
6. Dette, G.A., Knothe, H., Koulen, G., *Drugs Exp. Clin. Res.*, **13**, 567-576 (1987)
7. Chu, S.Y., Deaton, R., Cavanaugh, J., *Antimicrob. Agents Chemother.*, **36**, 147-1 150 (1992)
8. Ferrero, J.L., Bopp, B.A., Marsh, K.C., Quigley, S.C., Johnson, M.J., *et al*., *Drug. Metab. Dispos.*, **18**, 441-446 (1990)
9. Chu, S.Y., Sennello, L.T., Sonders, R.C., *J. Chromatogr*., **571**, 199-208 (1991)
10. Lerner, F.E., Caliendo, G., Santagada, V., Santana, G.S.M, Moraes, M.E.A, De Nucci, G., *Int. J. Clin. Pharmacol. Ther*., **38**, 345-354 (2000)
11. Chu, S., Park, Y., Locke, C., Wilson, D.S., Cavanaugh, J.C., *J. Clin. Pharmacol*., **32**, 32-6 (1992)
12. Chu, S., Wilson, D.S., Deaton, R.L., Mackenthun, A.V., Eason, C.N., Cavanaugh, J.H., *J. Clin. Pharmacol*., **33**, 719-26 (1993)
13. Chu, S.Y., Granneman, G.R., Pichotta, P.J., Decourt, J.P., Girault, J., Fourtillan, J.B., J. *Clin. Pharmacol.*, **33**, 480-5 (1993)
14. Chu, S.Y., Wilson, D.S., Guay, D.R., Craft, C., J. *Clin. Pharmacol.*, **32**, 1045-9 (1992)
15. Taninaka, C., Ohtani, H., Hanada, E., Kotaki, H., Sato, H., Iga, T., *J. Chromatogr. B. Biomed. Sci. Appl.*, 738, 405-11 (2000)
16. Sastre Torano, J., Guchelaar, H.J., *J. Chromatogr. B. Biomed. Sci. Appl*., **720**, 89-97 (1998)
17. Wust, J., Hardegger, U., *Chemotherapy*, **39**, 293-6 (1993)
18. Edlund, C., Alvan, G., Barkholt, L., Vacheron, F., Nord, C.E., *J. Antimicrob. Chemother.*, **46**, 741-9 (2000)
19. Benet, L.Z., *Transplant. Proc*., **30**, 1650-1 (1998)
20. Farolfi, M., Powers, J.D., Rescigno, A., *Pharmacol Res*. **39**, 1-4 (1999)

21. Benet, L.Z., *Transplant. Proc*. **31**, 7S-9S (1999)

5.3 Estudo 3

A PK/PD approach on the effects of clarithromycin against oral and nasal microbiota of healthy volunteers.

Ana Paula Del Bortolo Ruenis, PharmD, MS, CCRP[1]
Francisco Carlos Groppo, DDs, PhD[2]
Roberta Pessoa Simões, PharmD, PhD[3]
Luiz Madaleno Franco, PharmD, MS[4]
Pedro Luiz Rosalen, PharmD, PhD[2]
Ronilson Agnelo Moreno, PhD[5]
Eduardo Abib Júnior, M.D. M. Sc[5].

[1] Certified Clinical Research Professional (SoCRA), Ph.D student, Area of Pharmacology, Anesthesiology and Therapeutics - FOP/UNICAMP, Reseacher of the Bioequivalence Studies Unit - FOP/UNICAMP.

[2] Professor of Pharmacology and Reseacher of the Bioequivalence Studies Unit - FOP/UNICAMP.

[3] Reseacher of the Bioequivalence Studies Unit - FOP/UNICAMP.

[4] Professor of UNIMEP and Reseacher of the Bioequivalence Studies Unit - FOP/UNICAMP

[5] Reseacher of Synchrophar Assessoria e Desenvolvimento de Projetos Clínicos.

Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba
Piracicaba, SP, Brasil

State University of Campinas – UNICAMP
School of Dentistry of Piracicaba
Piracicaba, SP, Brazil

Correspondence:
Prof. Pedro Luiz Rosalen; Av. Limeira, 901; 13414-903 – Piracicaba, SP; Brazil
E-mail: rosalen@fop.unicamp.br

Abstract

The pharmacokinetics of clarithromycin (CLR) and its effects on oral and nasal microbiota in healthy volunteers were assessed in an open, randomized, two-period crossover design. A single 500 mg oral dose of CLR (Group 1 - Merck® and Group 2 - Klaricid®) was administered to all volunteers observing 1-week interval between doses. Blood samples were collected from pre-dose to 24 h. Plasmatic concentrations of CLR were quantified by LC-MS-MS method. Saliva and nasal mucosa swabs were obtained previously and after 1.33, 2, 6 and 12 hours of drug administration. PK parameters were determined to estimate T>MIC, % T>MIC and AUC_{0-24}/MIC ratios. Total microorganisms', staphylococci and streptococci counts were obtained in different culture media. No statistically significant differences were observed between the two formulations (ANOVA, $p>0.05$) regarding CLR plasmatic concentrations, AUC_{0-24} and C_{max}. Total microorganism (nasal), staphylococci and streptococci counts did not show statistical differences (Wilcoxon, $p>0.05$) between the two groups during each sampling time. Considering each group, no statistically significant differences were found among 1.33, 2, 6 and 12 hours, but differed from pre-dose counts (Friedman, $p<0.05$). Saliva total microorganism counts of Group 2 showed no additional significant decrease (Friedman, $p<0.05$) after 1.33 hours. Group 1 total microorganisms counts statistically decreased after 6 hours (Friedman, $p<0.05$). No significant correlation between PK/PD parameters and changes in $\log_{10}$ CFU/mL (r^2 ranging from -0.51 to 0.59, Spearman's test) was observed. This PK/PD model appears to be a better predictor of *in vivo* CLR efficacy, when compared to usual *in vitro* models.

Descriptors: clarithromycin, PK/PD, T>MIC, streptococci, staphylococci.

Introduction

It has been stated that the MIC (minimum inhibitory concentration) is the only satisfactory *in vitro* predictor of the intrinsic activity of antibiotics, providing a simple measure of concentration that can be directly compared to the concentrations of an antibiotic in blood, body fluids or tissues.[1)] However, MIC is an *in vitro* measurement carried out against a single bacterium, and even modern methods that employs antibiotic diffusion in agar as the Etest (Biodisk, Sweden) may have their results influenced by variables as inoculum density, composition of the medium, reading of the test, etc.[2)]

Other predictor of antimicrobial efficacy is the time kill assay.[3,4)] A time-kill study using concentrations of 0.25 to 8 times the MIC and an initial inoculum of approximately 10^5 CFU/ml showed that clarithromycin (CLR) elicited a concentration-independent bacteriostatic effect against *H. influenzae* and *S. aureus* at concentrations at least two times the MIC.[5)]

In vitro models simulating serum antimicrobial concentrations provide more information about the activity of an antibiotic than MICs or traditional time-kill methods.[6)] When an *in vitro* system and a validated animal model were compared, similar relationships between %T>MIC and effects were demonstrated. However, it is still premature to assume that an antibiotic's efficacy could be explained only by the use of *in vitro* conditions.[7)]

Macrolide antibiotics, including clarithromycin and its active metabolite, are bacteriostatic agents that inhibit protein synthesis by binding reversibly to 50S ribosomal sub-units of sensitive microorganisms.[8,9)] Its antimicrobial activity *in vivo* is enhanced by forming its active metabolite 14-hidroxiclarithromycin (14-OH), which shows synergistic activity against various pathogens including *Haemophyllus influenzae*, *Moraxella catarrhalis*, *Legionella* sp. and also streptococci and staphylococci.[10)]

Measurement of CLR and its active metabolite 14-OH in body fluids can be performed using high performance liquid chromatography (HPLC) techniques with electrochemical detection, as previously described, [11,12,13)] Reversed phase liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) was used to quantify the parent compound and its active metabolite. This method requires a simple extraction procedure, also reducing the analysis time. [13)]

Oral daily recommended dosage to adult patients is usually 250 to 500 mg BID, during 7 to 14 days and 7.5 mg/kg BID (up to 250 mg/day), during 5 to 10 days for pediatric patients depending on the infection severity and the etiological agent.[14,15)]

In Dentistry, CLR usually has replaced erythromycin as an alternative to penicillin-allergic patients, especially in the bacterial endocarditis prophylaxis.[14,15)] *Viridans* streptococci (alpha-hemolytic streptococci) and *S. aureus* are the most common cause of endocarditis following dental or oral procedures, upper respiratory tract procedures, bronchoscopy with a rigid bronchoscope, surgical procedures involving the respiratory mucosa, and esophageal procedures.[16,17,18)] The standard bacterial endocarditis prophylaxis regimen includes 500 mg single dose of CLR (15 mg/kg for children), one hour before the procedure, for individuals who are allergic to penicillins.

The establishment of an "ideal" model to explain how pharmacokinetics/pharmacodynamics (PK/PD) properties could be applied to select the most appropriate dose and dose regimens is still a matter of concern. It would be useful to elucidate an antibiotic's concentration-time-course effect through an *in vitro* method that could minimize the use of experimental animals and human trials in the early stages of drug development.

For a wide number of antibiotic groups (e.g. aminoglycosides, fluoroquinolones, metronidazole, daptomycin, ketolides and amphotericin), the C_{max}/MIC and $AUC_{0\text{-}24}$/MIC ratios are the most important aspects to be observed, probably because of their marked concentration-dependent killing effect. Other antibiotics (β-lactams, vancomycin, erythromycin, clindamycin and tetracyclines) have little or no concentration-dependent effects on bacteria. These agents do not need high concentration peaks (C_{max}) and their dosages must be optimized taking into account %T>MIC (the duration of time the concentration exceeds the MIC) values. Both %T>MIC and AUC_{24h}/MIC influence the clinical efficacy of CLR and azithromycin, but not erythromycin.[19)]

The aim of this study was therefore to compare the pharmacokinetic and the efficacy of two CLR formulations against oral and nasal microbiota.

Materials and methods

CLR formulations

GROUP 1 - Claritromicina Merck® (500 mg of CLR, produced by Merck S.A Indústrias Químicas) and GROUP 2 - Klaricid® (500 mg of CLR, produced by Abbott Laboratórios do Brasil, Ltda) were supplied as tablets.

Clinical protocol

This study enrolled 20 healthy volunteers (50% of males), ranging in age from 18 to 45 years, in weight from 50 to 90 kg (62.52 ± 8.99 kg) and in height from 1.50 to 1.80 m (1.63 ± 0.08 m). The clinical trial was conducted according to the I.C.H Harmonized Tripartite Guideline for Good Clinical Practice (1996), the Declaration of Helsinki (1965) and Resolutions 196 (1996) and 251 (1997) – National Health Council, Health Ministry, Brazil.

The subjects were informed of the purpose, protocol and risks of the study. Individuals using any medications 14 days prior to or throughout the study, including over-the-counter products, and hypersensitivity to CLR were excluded. The FCM/UNICAMP Ethics Committee approved the protocol (# 190/2001) and all subjects provided written informed consent.

The volunteers were hospitalized and fasted 10 hours before dosing. The subjects received a single oral dose of either CLR Merck® or Klaricid® in an open, randomized, two-period crossover design with 1-week wash-out period between doses. Standardized meals were given 4, 8, 10 and 12 hours after drug administration. No other food was permitted during the sampling period. Except xanthine-containing drinks including tea, coffee, and cola derivatives, liquid consumption was permitted *ad libitium* 2 hours after drug administration. All the volunteers were instructed not to perform tooth brushing and dental flossing from the beginning of the fasting period to the final saliva sampling (12 h).

Non-stimulated saliva (± 1 ml) and nasal secretion samples were collected at the following time periods: pre-dose, 1.33, 2, 6 and 12 hours after drug administration. Blood samples were collected at pre-dose, 0.33, 0.66, 1, 1.33, 1.66, 2, 2.5, 3, 4, 6, 8, 10, 12,

16, 20 and 24 h after the administration, to assess AUC_{0-24}. The blood samples were centrifuged at 4550 X *g* for 5 minutes (4°C) and plasma was stored at -20°C for further LC-MS-MS analysis.[13)] Saliva aliquots (200 µL) were transferred to eppendorf tubes containing 1.8 ml Stuart's transport medium and immediately stored in dry ice.

Nasal secretion samples were obtained by introducing a sterile swab immersed in 200 µL of 0.9% NaCl solution into nasal cavity; the swab tips were cut into eppendorf tubes containing 1.8 ml Stuart's transport medium and immediately stored in dry ice. Further, these samples were sonicated (5% amplitude, six 10-min pulses, 5 seconds interval between each pulse, 32W potency, Vibra Cell 400W, Sonics & Materials Inc, Danbury, CT, USA). Saliva and nasal samples were diluted and 50 µL of each sample were logarithmically plated by an automatic spiral plater (Whitley Automatic Spiral Plater, Don Whitley Scientific Limited, Shipley, UK) on different culture media, in duplicate. Total microorganisms were recovered from 5% Blood Sheep Agar (BSA) and streptococci from Mitis Salivarius Agar (MSA) after 48 hours of incubation at 37°C, 10% CO_2. BSA plates were additionally incubated during 24 hours at aerobic conditions. Staphylococci were recovered from nasal secretion samples after dispersion on Salt Manitol Agar (SMA) plates incubated at aerobic conditions, during 24 hours. The number of colony forming units per ml (CFU/ml) was established by manual counting device.

Pharmacokinetic and statistical analysis

Mean $T_{1/2}$ (elimination half-life) and mean Vd (volume of distribution) were obtained from plasma concentration-time data. AUC_{0-24} (area under the plasma concentration-time curve from baseline to the last measurable or quantifiable time point) was determined from CLR concentrations from pre-dose to 24 h time period. These parameters were determined by software programs (WinNonlin Professional Network edition, version 1.5m; Bioequivalence Program for Two-Period Cross-Over Studies, version 3.4). Also, T>MIC (time above minimum inhibitory concentration) and %T>MIC (time above minimum inhibitory concentration, expressed as percentage) were calculated by using the following equations) [1)]:

T>MIC = ln (dose/Vd x MIC) x ($T_{1/2}$/ln2)

%T>MIC = ln (dose/Vd x MIC) x ($T_{1/2}$/ln2) x (100/DI)

where:

$T_{1/2}$: elimination half-life (h);

Vd: volume of distribution (l);

DI: dose interval, which was considered in the present study as both 12 h and 24 h.

The MIC_{90} values considered for the calculation of %T>MIC were 2 µg/ml for *S. aureus* and ≤0.25 µg/ml (susceptible), 0.5 µg/ml (intermediate resistant) and ≥1.0 µg/ml (resistant) for streptococci. [20,21,22,23)]

The individual changes in bacterial counts were calculated by subtracting the $\log_{10}$ CFU/ml of bacterial counts obtained at pre-dose time point from $\log_{10}$ CFU/ml of bacterial counts obtained at 1.33 h, and expressed as percentage. Differences between the other sequences (1.33 and 2 h, 2 h and 6 h, 6 h and 12 h) were similarly obtained.

Differences between groups 1 and 2 regarding microbiological counts or PK/PD parameters at the same time periods were analyzed by Wilcoxon test (5 % of significance level). Comparisons among time periods regarding microbiological counts or PK/PD parameters within groups were assessed by Friedman test (5% of significance level). Analysis of variance (ANOVA) was performed to verify differences in plasmatic concentration between the groups (log transformed values).

Spearman's correlation coefficient (r^2) test was used in order to verify correlation between PK/PD parameters and changes in $\log_{10}$ CFU/ml of bacterial counts.

Results

The CLR plasmatic concentrations (mean±SD) obtained at the nasal and saliva sampling time points were 2.19±0.87, 2.28±0.87, 1.39±0.51 and 0.46±0.17 µg/ml (Group 1) and 2.24±0.70, 2.32±0.68, 1.64±1.08 and 1.02±2.52 µg/ml (Group 2) after 1.33, 2, 6 and 12 hours, respectively. No statistically significant differences were observed between the two formulations (P > 0.05). CLR concentrations obtained from all blood samples are illustrated in Figure 1.

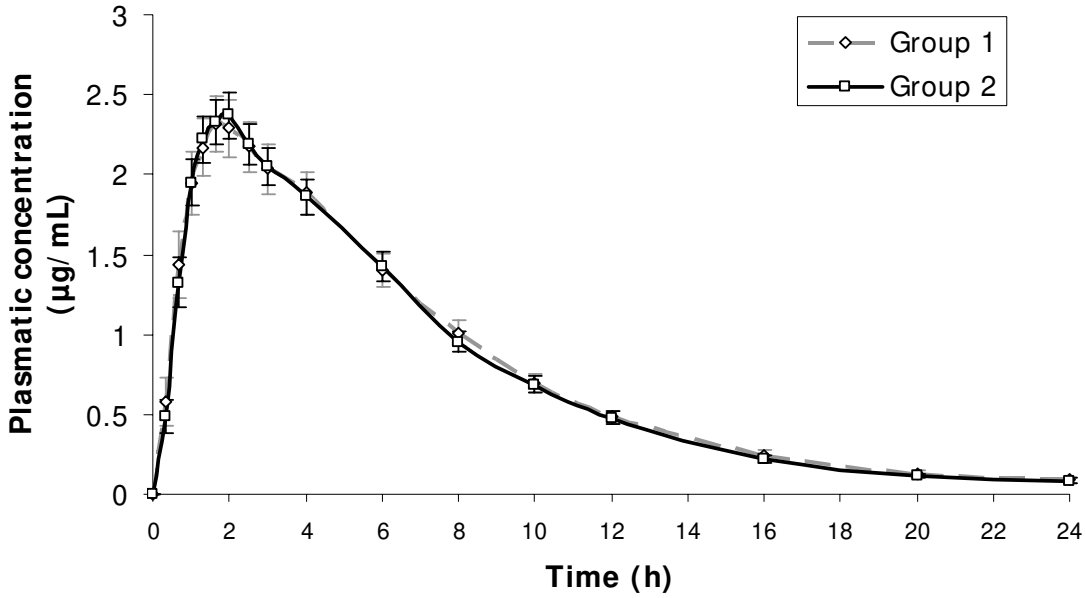


Figure 1. Concentration versus time curves. Mean plasmatic concentrations (µg/ml ± SD) of CLR obtained from 20 volunteers, during the saliva and nasal secretion-sampling period.

Total microorganism and staphylococci counts from nasal samples did not show statistically significant differences (Wilcoxon test, $p>0.05$) between the two groups during each sampling time. Considering each group, no statistically significant differences were found among 1.33, 2, 6 and 12 hours. However, these periods showed significant differences when compared to the pre-dose counts (Friedman test, $p>0.05$). Thus, no additional significant decrease on total microorganism or on staphylococci counts was observed after 1.33 h.

Figure 2 shows microbiological recovery (total and staphylococci counts) data obtained from nasal secretion sampling.

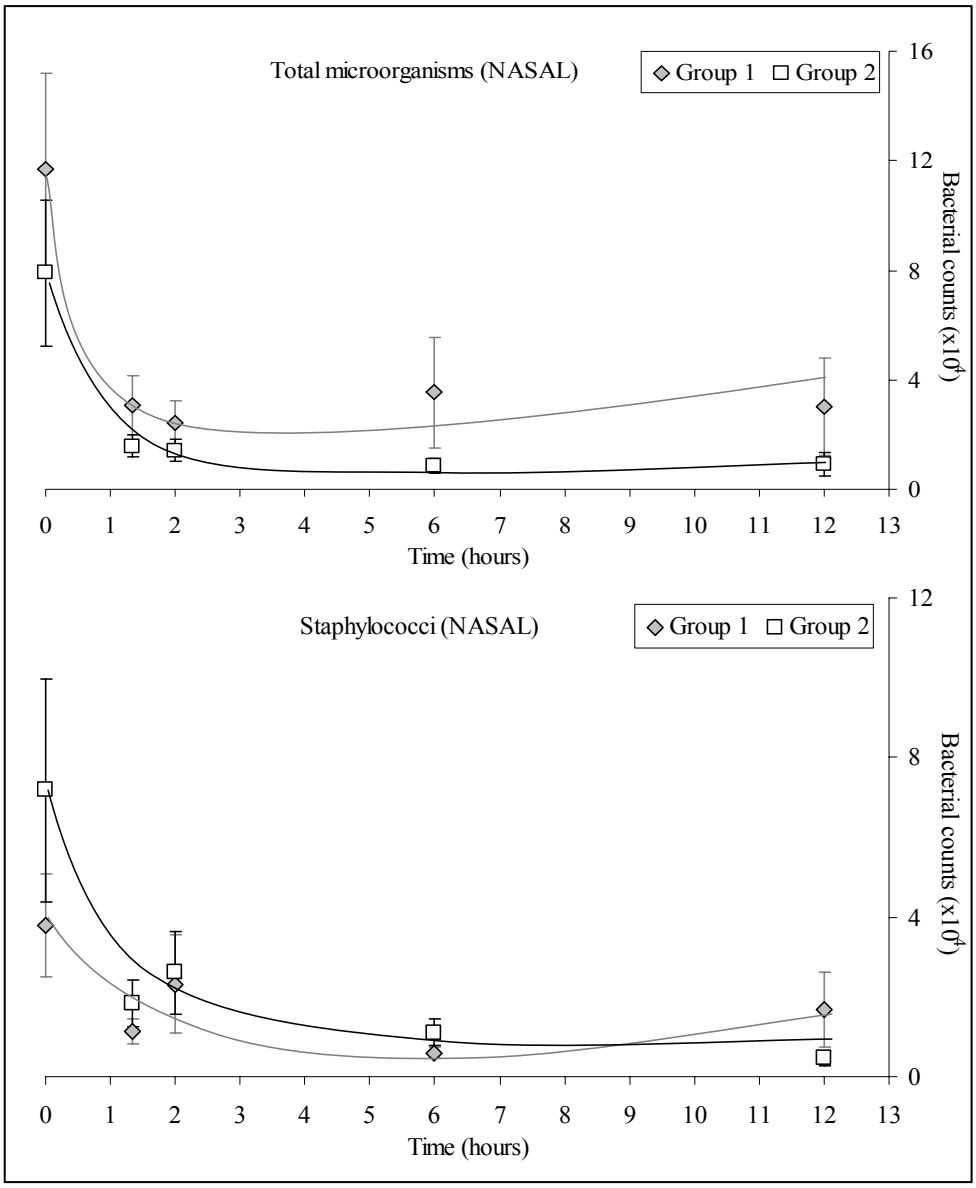


Figure 2. Mean values (± S.E.M.) of total microorganisms and staphylococci counts (UFC/mlx10^4) from nasal samples of 20 volunteers after the administration of CLR formulations.

Total microorganism and streptococci counts from saliva samples did not show statistically significant differences (Wilcoxon test, $P > 0.05$) between the two groups during each sampling time. Considering streptococci counts within each group, no statistically significant differences were found among 1.33, 2, 6 and 12 hours but these time periods exhibited statistical differences when compared to the counts of the zero time point

(Friedman test, $P > 0.05$).

Saliva total microorganism counts of Group 2 showed no additional significant decrease (Friedman test, $P < 0.05$) after 1.33 h. However, considering Group 1, statistically significant decrease was found only after 6 hours ($P > 0.05$).

Figure 3 shows microbiological recovery (total and streptococci counts) data obtained from saliva sampling.

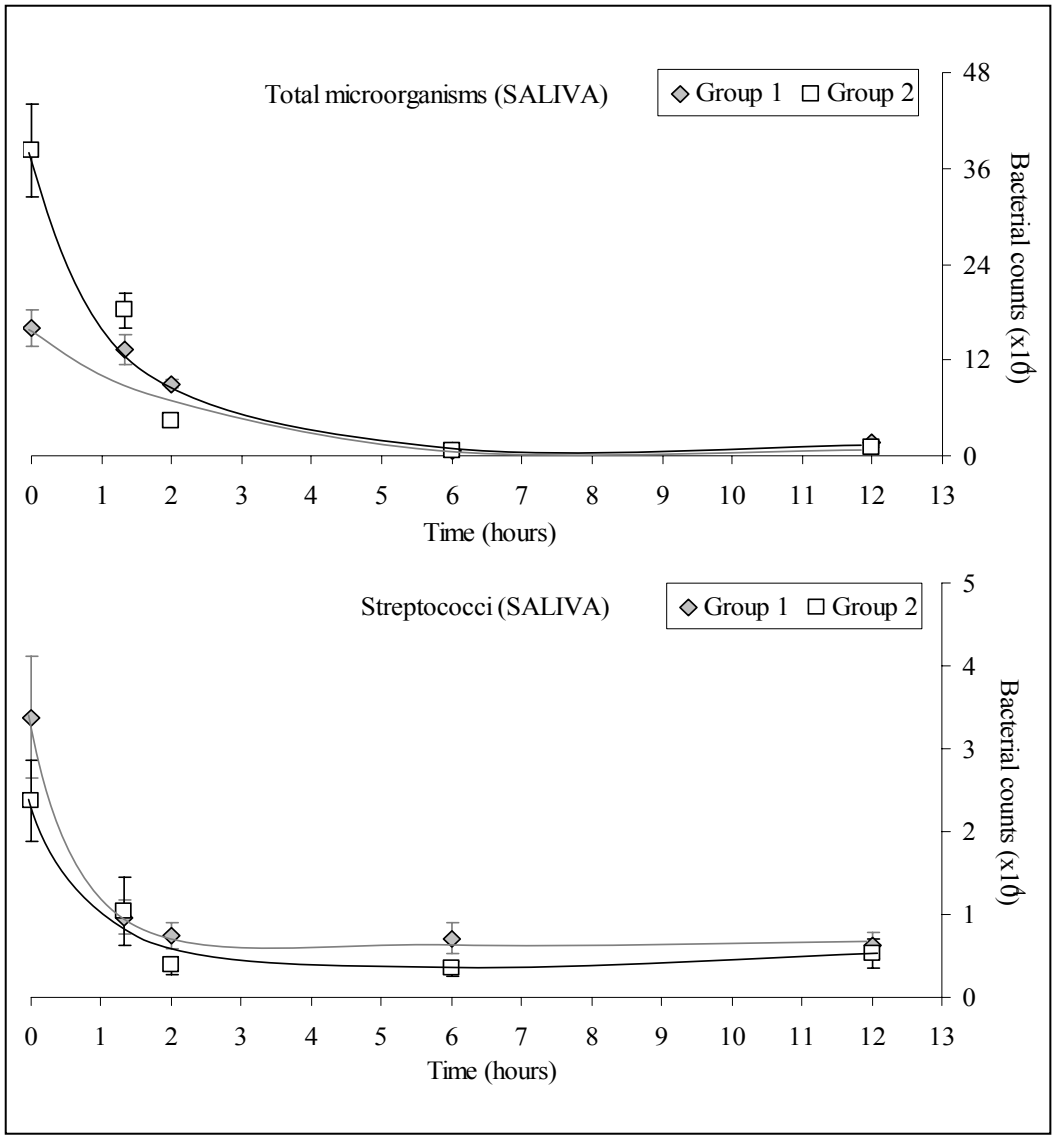


Figure 3. Mean values (± SD) of total microorganisms and streptococci counts (ufc/mlx10^4) from saliva of 20 volunteers after the administration of CLR formulations.

Mean cumulative changes in bacterial counts (log_{10} CFU/ml) are demonstrated at Table 1.

Table 1. Mean (±SD) cumulative changes in bacterial counts (log_{10} CFU/ml) after the administration of CLR formulations.

		% cumulative changes (log_{10} CFU/ml)	
	Time points-interval (h)	**Group 1**	**Group 2**
Saliva total microorganisms	0 – 1.33	36.3	36.3
	1.33 – 2.0	78.1	77.7
	2.0 – 6.0	97.6	98.0
	6.0 – 12.0	92.4	93.5
Saliva streptococci	0 – 1.33	72.7	71.6
	1.33 – 2.0	82.4	73.5
	2.0 – 6.0	86.0	90.1
	6.0 – 12.0	82.0	77.6
Nasal total microorganisms	0 – 1.33	67.7	48.4
	1.33 – 2.0	67.2	76.4
	2.0 – 6.0	73.5	71.5
	6.0 – 12.0	82.6	88.6
Nasal staphylococci	0 – 1.33	63.4	58.7
	1.33 – 2.0	74.7	84.0
	2.0 – 6.0	90.6	80.4
	6.0 – 12.0	86.4	88.5

The PK/PD indexes (T>MIC, %T>MIC and AUC_{0-24}/MIC) used to correlate *in vitro* and *in vivo* efficacy were described in Table 2.

Table 2. Mean (±SD) AUC_{0-24}/MIC, T>MIC and %T>MIC values after the administration of CLR formulations, considering 12 h or 24 h-dose interval, calculated from pharmacokinetic data obtained from 20 volunteers.

DI (h)	PK/PD	Group 1	Group 2
12	T>MIC 0.25	14.45 (±1.69)	14.50 (±1.92)
	T>MIC 0.5	10.03 (±0.84)	10.11 (±1.04)
	T>MIC 1.0	5.61 (±1.15)	5.73 (±1.03)
	T>MIC 2.0	1.19 (±2.17)	1.35 (±1.91)
	%T>MIC 0.25	120.43 (±14.08)	120.8 (±16.04)
	%T>MIC 0.5	83.59 (±7.03)	84.29 (±8.64)
	%T>MIC 1.0	46.75 (±9.59)	47.78 (±8.57)
	%T>MIC 2.0	9.91 (±18.06)	11.27 (±15.93)
24	%T>MIC 0.25	60.22 (±7.04)	60.40 (±8.02)
	%T>MIC 0.5	41.80 (±3.51)	42.14 (±4.32)
	%T>MIC 1.0	23.37 (±4.79)	23.89 (±4.28)
	%T>MIC 2.0	4.95 (±9.03)	5.64 (±7.96)
	AUC_{0-24}/MIC 0.25	73.10 (±24.77)	73.18 (±20.08)
	AUC_{0-24}/MIC 0.5	36.55 (±12.38)	36.59 (±10.04)
	AUC_{0-24}/MIC 1.0	18.28 (±6.19)	18.30 (±5.02)
	AUC_{0-24}/MIC 2.0	9.14 (±3.1)	9.15 (±2.51)

Spearman's correlation coefficient (r^2) test did not show any significant relationship between PK/PD parameters and changes in $\log_{10}$ CFU/mL (r^2 ranging from -0.51 to 0.59) considering each period of time in relation to pre-dose counts for all bacterial samples.

Discussion

A high performance liquid chromatography (HPLC) with electrochemical detection method to evaluate plasma concentrations of CLR was previously described.[24] The same instrumentation was used to assay its broncopulmonary, plasma pharmacokinetics,[25] and in epithelial lining fluid.[26] In the present study, we validated a LC-MS-MS assay to quantify CLR plasmatic concentrations which showed linearity, specificity, precision, accuracy and a very low limit of quantification (0.02 μg/mL) by using a simple extraction procedure and reduced analysis time.

The results obtained in the present study were similar to those reported in previous studies using volunteers submitted to a single 500 mg oral dose of CLR. These studies reported concentrations of 2.72 ± 0.87 after 2 h of drug administration, 1.21 ± 0.34 after 7 h and 0.73 ± 0.35 mg/l after 12 hours.[27] Data obtained from Brazilian volunteers showed CLR plasmatic concentrations ranging from 2.7 μg/ml to 2.9 μg/ml, after 1.5 h.[13]

In the present study, both CLR groups demonstrated efficacy against the studied microorganisms, since a decrease in total and staphylococci counts was observed 1.3 hours after drug administration, which were maintained over the sampling period. Even after 12 hours, a reduction of 85% in total and staphylococci counts could be observed.

After 12 h of drug exposure, saliva total bacterial counts and streptococci counts were reduced to 93% and 80% of the initial counts, respectively. Around 36% of total counts were reduced in 1.3 hours, while streptococci counts reduced 72% in the same period of time. The lowest total and streptococci recoveries are observed during the interval between 2 hours (corresponding to the observed T_{max}) and 6 hours (after two elimination half-lives) of CLR administration. This verified activity against oral microorganisms could be compared to the efficacy of typical bactericidal agents, such as beta-lactams.

Although salivary concentrations were not evaluated in our work, the saliva/serum ratio (0.8) previously reported could partially explain the significant decrease observed in streptococci saliva counts right after 1.33 hours, since related serum concentrations required are $\leq$0.25 μg/ml against susceptible streptococci, 0.5 μg/ml against

intermediate resistant strains and ≥ 1.0 μg/ml against resistant ones.[28] However, MIC is just an *in vitro* measurement, which is influenced by many variables, and it is not a good predictor of *in vivo* efficacy.[2] It has been stated that C_{max} could directly influence the overall efficacy of antimicrobial agents with high concentration-dependent killing. Our preliminary observations could precipitately suggest that C_{max}/MIC would be the best predictor of CLR efficacy in this model, since a very significant decrease in bacterial counts was observed in 1.33 hour and, after that, no additional significant decrease was observed. However, this fact could mean that no regrowth was observed within 1.33 to 12 hours when low CLR concentrations were achieved showing a markedly concentration-independent killing effect. This observation agrees with other reports which state that the most appropriate PK/PD parameters for macrolides are the AUC_{24h}/MIC ratio and T>MIC.[2]

A non-compartmental *in vitro* model to assay CLR activity against *S. pneumoniae* isolates with MIC values ranging from 0.12 to 128.0 μg/ml, reproducing an oral dosage of 500 mg, twice a day, in order to simulate free-drug serum C_{max} of 2 μg/ml was previously related.[29] The authors demonstrated that %T>MIC values higher than 90% and AUC_{24h}/MIC ratio higher than 61 resulted in bacterial eradication, while %T>MIC values ranging from 40 to 56% (AUC_{24h}/MIC ratio ≥30.5 to 38) showed a 1.2 to 2.0 $\log_{10}$ CFU/ml decrease at 24 h. %T>MIC values of ≤8% and AUC_{24h}/MIC ratio ≤17.3 resulted in a static effect or bacterial regrowth. A review over a series of clinical studies that used macrolides to treat bacterial otitis media showed a clear dose-effect relationship between %T>MIC and clinical efficacy with a maximum of 90% of cure achieved when T>MIC was around 50%.[2]

In our study, %T>MIC (considering both DI) values and AUC_{24h}/MIC ratios achieved with MICs of 0.25 and 0.5 μg/ml exceeded previously described ratios required to eradicate or decrease bacterial counts.[29] Thus, considering pathogens within this MIC range a high probability of cure could be expected *in vivo*. The comparison between both AUC_{24h}/MIC ratio %T>MIC considering MICs of 1.0 and 2.0 μg/ml and data previously reported could suggest an *in vivo* static effect or bacterial regrowth.[29] This extrapolation from an *in vitro* result to *in vivo* efficacy does not consider many other aspects regarding

drug properties and host conditions.

The post-antibiotic effect (PAE), e.g. the delay in bacterial regrowth following the removal of the antimicrobial agent after an initial challenge, is influenced by the drug concentration, the time that microorganisms are exposed to the antibiotic and its mechanism of action. A low PAE ranging from 1.5 to 3 h *in vitro* was related for CLR and 14-OH respectively, against anaerobes and *S. aureus*.[30] In contrast, azithromycin shows a marked PAE *in vivo* due to a high tissue binding and slow leakage from phospholipids or other intracellular components.[19] Thus, CLR PAE could not explain the results observed in the present study.

All macrolides, including CLR, are readily taken up by polymorphonuclear leucocytes (PMNLs), monocytes, lymphocytes and alveolar macrophages, achieving high intracellular concentrations (about 8-fold higher than the serum concentration). When the drug is transferred to the sites of infection, bacteria are exposed to high bactericidal concentrations of the antibiotic. The host defense system work simultaneously to achieve the maximum bacterial eradication.[31] In the present study, the host defense system work of the healthy volunteers enrolled could certainly contribute to reduced bacterial counts verified.

CLR is metabolized *in vivo* to its active metabolite, 14-OH. *In vitro* studies demonstrated that the metabolite alone is as potent as the parent compound against *S. pneumoniae* susceptible strains (MIC= 0.125 mg/L). Synergy was demonstrated with the combination CLR/14-OH (1:1 and 2:1 ratios) resulting in lower MICs values (0.063 mg/L).[31] Studies have reported that 14-OH plasmatic concentrations can reach up to 40% to 50% of the parent compound.[13] These data suggest that the metabolite parameters should be considered in the PK/PD approaches, to better predict the clinical efficacy of CLR.

In the present study, any significant correlation between PK/PD parameters and changes in $\log_{10}$ CFU/mL was found. In contrast to other *in vitro* reports, unable to estimate host conditions, the PK/PD model presented herein provides more information about the *in vivo* characteristics of the antimicrobial agent, and could be a better predictor of antibiotics efficacy. This approach should include the PK/PD parameters considering parent drug and the metabolite, plus the *in vivo* estimation of PAE, which could be applied simultaneously

to phase I clinical trials and be a better predictor of antibiotics efficacy in the early stages of drug development.

Acknowledgment

This study was supported by a grant from Merck S.A Indústrias Químicas. The author Ana Paula Del Bortolo Ruenis was supported by a scholarship provided by CNPq, Brazil.

References

1. Turnidge, J.D., *CID*, **27**, 10-22 (1998).

2. Frimodt-Moller, N., *Int. J. Antimicrob. Agents*, **19**, 333-339 (2002).

3. Credito, K.L., Ednie, L.M., Jacobs, M.R., Appelbaum, P.C., *Antimicrob. Agents Chemother.*, **43**, 2027-2031 (1999).

4. Visalli, M.A., K.L., Jacobs, M.R., Appelbaum, P.C., *Diagn. Microbiol. Infect. Dis.*, **28**, 131-137 (1997).

5. Burgess, D.S., Hastings, R.H. Horan, J.L., *Ann. Pharmacother.*, **33**, 1262-1265 (1999).

6. Alou, L., Sevillano, D., Bugella, J.H., Fuentes, F., Gomez-Lus, M.L., Prieto, J., *Int. J. Antimicrob. Agents*, **17,** 365-370 (2001).

7. Bonapace, C.R., Friedrich, L.V., Bosso, J.A., White, R.L., *Antimicrob. Agents Chemother.*, **46**, 3574-3579 (2002).

8. Kakegawa, T., Ogawa, T., Hirose, S., *Chemotherapy*, **36**, 123-128 (1988).

9. Kakegawa T., Hirose S., *Chemotherapy*, **38**, 317-323 (1990).

10. Jones, R.N., Erwin, M.E., Barrett, M.S., *Eur. J. Clin. Microbiol. Infect. Dis.*, **9**, 846-848 (1990).

11. Chu, S.Y., Deaton, R., Cavanaugh, J., *Antimicrob. Agents Chemother.*, **36**, 147-150 (1992).

12. Kim, Y.G., Kim, H.J., Kwon, J.W., Kim, W.B., Lee, M.G., *Int. J. Clin. Pharmacol. Ther.*, **39**, 356-361 (2001).

13. Lerner, F.E., Caliendo, G., Santagada, V., Santana, G.S.M, Moraes, M.E.A, De Nucci, G., *Int. J. Clin. Pharmacol. Ther.*, **38**, 345-354 (2000).

14. Dajani, A.S., Bawdon, R.E., Berry, M.C., *J. Am. Med. Ass.*, **277**, 1794-1801 (1997).

15. Taubert, K.A. & Dajani, A.S., *Am. Farm. Physician*, **57**, 457-468 (1998).

16. Cowper, T.R., *Dent. Clin. North Am.*, **40**, 611-647 (1996).

17. Dudkiewicz, B., Mikucki, J., Ostrowski, S., *Mater. Med. Pol.*, **28**, 49-56 (1996).

18. Benn, M., HAgelskjaer, L.H., Tvuede, M., *J. Intern. Med.*, **242**, 15-22 (1997).

19. Van Bambeke, F.,Tulkens., P.M., *Int. J .Antimicrob. Agents*, **18**, S17-S23 (2001).

20. Benson, C.A., Segreti, J., Beaudette, F.E., Hines, D.W., Goodman, L.J., Kaplan, R.L., Trenholme, G.M., *Antimicrob. Agents Chemother.*, **31**, 328-330 (1987).

21. Fernandes, P.B., Bailer, R., Swanson, R., Hanson, C.W., McDonald, E., Ramer, N., Hardy, D., Shipkowitz, N., Bower, R.R. and Gade, E., *Antimicrob. Agents Chemother.*, **30**, 865-873 (1986).

22. Chin, N.X., *Antimicrob. Agents Chemother.*, **31**, 463-466 (1987).

23. Floyd-Reising, S., Hindler, J.A., Young, Ls., *J. Antimicrob. Chemother.*, **31**, 640-642 (1987).

24. Chu, S.Y., Sennello L.T., Sonders, R.C., *J. Chromatogr.*, **571**, 199-208 (1991).

25. Patel, K.B., Xuan, D., Tessier, P.R., Russomanno, J.H., Quintiliani, R., Nightingale, C.H., *Antimicrob. Agents Chemother.*, **40**, 2375-2379 (1996).

26. Rodvold, K.A., Gotfried, M.H., Danziger, L.H., Servi, R.J., *Antimicrob. Agents Chemother.*, **41**, 1399-1402 (1997).

27. Wust, J., Hardegger, U., *Chemotherapy*, **39**, 293-6 (1993).

28. Soriano, F., Rodriguez-Cerrato, V., *J. Antimicrob. Chemother.*, **50**, 51-58 (2002).

29. Noreddin, A.M., Roberts, D., Nichol, K., Wierzbowski, A., Hoban, D.J., Zhanel, G.G., *Antimicrob. Agents Chemother.*, **46**, 4029-4034 (2002).

30. Jung, R., Messick, C.R., Pendland, S.L., Tesoro, E.P., Losendahl, K.J., Schriever, C.A., Danziger, L.H., *Antimicrob. Agents Chemother.*, **44**, 778-779 (2000).

31. Amsden, G.W., *Int. J. Antimicrob. Agents*, **18**, S11-S15 (2001).

32. Martin, S.J., Garvin, C.G., McBurney, C.R., Sahloff, E.G., *J. Antimicrob. Chemother.*, **47**, 581-587 (2001).

5.4 Estudo 4

The effects of the interactions between CYP3A4 and P-gp on the *in vitro* disposition of clarithromycin.

Ana Paula Del Bortolo Ruenis, PharmD., M.S, CCRP[1]
Leslie Z. Benet, PharmD., PhD[2]
Pedro Luiz Rosalen, PharmD, PhD[3]
Francisco Carlos Groppo, DDs, PhD [3]

[1] Certified Clinical Research Professional (SoCRA), PhD student, Area of Pharmacology, Anesthesiology and Therapeutics - FOP/UNICAMP, Researcher of the Bioequivalence Studies Unit - FOP/UNICAMP

[2] Professor of the Department of Biopharmaceutical Sciences, University of California at San Francisco (UCSF), USA

[3] Professor of FOP/UNICAMP and Researcher of the Bioequivalence Studies Unit - FOP/UNICAMP.

Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba
Piracicaba, SP, Brasil

State University of Campinas – UNICAMP
School of Dentistry of Piracicaba
Piracicaba, SP, Brazil

Correspondence:
Prof. Pedro Luiz Rosalen; Av. Limeira, 901; 13414-903 – Piracicaba, SP; Brazil
E-mail: rosalen@fop.unicamp.br

Abstract

CYP3A4-transfected Caco-2 cells were used as an *in vitro* system to predict the importance of drug metabolism and transport of clarithromycin (CLR), in the presence of cyclosporine (10 μM CsA, a CYP3A4/P-gp inhibitor) or 200 nM GG918 (an inhibitor of P-gp and not CYP3A4). The donor solutions were prepared adding CLR to a final concentration of 5.0 μg/mL. An aliquot of 100 μL was taken from the receiver side after 30 minutes, 1 h and 2 h of incubation. CLR transport in the basolateral (B) to apical (A) direction was higher then the A to B direction, showing the P-gp activity in the efflux of CLR in the control group. In the presence of the P-gp inhibitor (GG918, 200 nM) a significant decrease in the B to A CLR transport is observed. The B to A transport was higher in the first time point (30 minutes) and decreased after 1 and 2 h, showing concentrations similar to the control. In the presence of the P-gp and CYP3A4 inhibitor (CsA 10 μM) a significant decrease in the B to A CLR transport was observed. The B to A transport was higher in the first time point (30 minutes) and decreased after 1 and 2 h. The CLR active metabolite (14-OH), formed after incubation of CLR in CYP3A-transfected Caco-2 cells, was mostly transported in the A to B direction, demonstrating the passive absorption of CLR and its intestinal first pass-extraction by CYP3A4. The data obtained with CLR from CYP3A4-transfected Caco-2 cells suggests that multiple transporters could be involved in the disposition of this drug, since the effect of P-gp on metabolism was not similar to other drugs considered as typical P-gp substrates. Also, this effect appears to be time-dependent, and further studies with different time points and different concentrations of both drug and inhibitors are needed to completely elucidate the role of intestinal intracellular enzymes and transporters as absorption barriers to clarithromycin.

Descriptors: clarithromycin, CYP3A4, P-gp, CYP3A4 Caco-2 cells.

Introduction

The systemic availability of an orally administered drug depends on physical and biological factors, including the dissolution and disintegration properties of the drug formulation, solubility of the drug in the gastrointestinal tract, plasma membrane permeability and the susceptibility of the molecule to plasma membranes transporters and biotransformation enzymes, present in the gut and liver (Hall *et al.*, 1999). Intestinal drug absorption can be significantly limited by metabolizing enzymes and efflux transporters in the gut (Benet *et al.*, 1996b).

This intestinal first-pass extraction occurs due to the activity of CYP3A4, the most abundant oxidative cytochrome P 450 drug metabolizing enzyme present in the intestine (Benet *et al.*,1996a). CYP3A4 substrates are also substrates for the multidrug resistance transporter P-glycoprotein (P-gp), a member of the ATP-binding cassette family of transporter proteins. Both P-gp and CYP3A4 are located in the columnar epithelial cells lining the intestinal lumen (Cummins *et al.*, 2001).

In the enterocyte, the spatial separation of P-gp, transversing the apical plasma membrane, and that of CYP3A4, located in the endoplasmatic reticulum, could explain the role of these proteins in limiting oral bioavailability. Drugs absorbed into the intestinal epithelium can interact with P-gp and be extruded back into the intestinal lumen, limiting the entry of drugs into the cell (Ambudkar *et al.*, 1999; Cummins *et al.*, 2001). The repeated process of diffusion and the active counter transport, mediated by P-gp, prolongs the intracellular residence time of the drug, which is exposed to the CYP3A4 activity. The interplay between CYP3A4 and P-gp in the intestine results in enhanced drug metabolism and significant decrease in intestinal drug absorption (Cummins *et al.*, 2002).

Macrolides have demonstrated interactions with P-glycoprotein (P-gp) and CYP3A4 in different studies (Pea & Furlanut, 2001; Prieto, 2001; Moore *et al.*, 2002; Williams *et al.*, 2002). Clarithromycin (CLR) is a large spectrum macrolide antimicrobial agent, similar to erythromycin against susceptible microorganisms. CLR oral bioavailability is 55%, because of its rapid first-pass metabolism (Chu *et al.*, 1992a) and its

activity is enhanced by the *in vivo* formation of the active metabolite 14-hidroxiclarithromycin (14-OH) (Jones *et al.*, 1990; Fraschini *et al.*, 1993).

Clinical reports showing the interaction between CLR and digoxin demonstrated that renal digoxin clearance to creatinine clearance in one patient was lower during the concomitant administration of CLR (0.64 and 0.73) than the one observed after cessation of the drug administration (1.30 ± 0.20) (Wakasugi *et al.*, 1998). In the same study, the authors evaluated that CLR, *in vitro*, inhibited the transcellular transport of digoxin from the basolateral to the apical (B to A) side in a concentration-dependent manner, and concomitantly increased the cellular accumulation of digoxin, using a kidney epithelial cell line (Wakasugi *et al.*, 1998).

When vinblastine (200 μg/kg) was administered intraperitoneally with each of the macrolide antibiotics (10 or 100 mg/kg) or with verapamil (25 mg/kg) once a day for 10 days in P388/ADR-bearing mice, combined effects of vinblastine with the macrolide antibiotics (erythromycin, CLR and YM17K) or verapamil were observed. CLR also increased the accumulation of both vinblastine and CsA in P388/ADR cells in a dose-dependent manner (Wang *et al.*, 2000). *In vivo* studies found that digoxin concentrations increased during concomitant administration of CLR (before, 0.838 ± 0.329 ng/mL and 1.36 ± 0.619 ng/mL after the CLR administration), and this effect was dose-dependent on CLR. The percentage increase in digoxin concentrations after the usual oral dose of clarithromycin (400 mg/d, for Japanese patients) is approximately 70% (Tanaka *et al.*, 2003).

Clinical drug interactions have demonstrated that CLR inhibits CYP3A4 activity. Mean area under the plasma concentration-time curves from 0 to 24 hours (AUC) of omeprazole in homozygous extensive metabolizer, heterozygous extensive metabolizer, and poor metabolizer groups were significantly increased by clarithromycin from 383.9 to 813.1, from 1001.9 to 2110.4, and from 5589.7 to 13098.6 ng x h/mL, respectively (Furuta *et al.*, 1999). Co-administration of clarithromycin and amprenavir significantly increased the mean amprenavir AUC(ss), C(max,ss), and C(min,ss) by 18, 15, and 39%, respectively. Amprenavir had no significant effect on the AUC(ss) of clarithromycin, but the median T(max,ss) for clarithromycin increased by 2.0 h, renal clearance increased by 34%, and the

AUC(ss) for 14-(R)-hydroxyclarithromycin decreased by 35% when it was given with amprenavir (Brophy *et al.*, 2000).

An *in vitro* model, combined with *in vivo* published data, predicted that clarithromycin at a clinically relevant unbound plasma concentration of 0.1 μM reduces the steady-state concentration of liver CYP3A4 to approximately 39% of initial levels, corresponding to 2.6-fold increases in the area under the plasma concentration-time curve of a co-administered drug that is eliminated exclusively by hepatic CYP3A4 metabolism for these compounds (Mayhew *et al.*, 2000).

Many other studies classifies CLR as a major CYP3A4 inhibitor (Sapone *et al.*, 2003; Mensa *et al.*, 2003; Yan *et al.*, 2002; Williams *et al.*, 2002; Moore *et al.*, 2002; Prieto 2001; Pea & Furlanut, 2001; Niemi *et al.*, 2001). However, the effects of the interplay between CYP3A4 and P-gp in the disposition of CLR have not been evaluated until the present. The goal of this study is therefore to identify the role of P-gp in determining the extent of CLR metabolism mediated by intestinal CYP3A4.

Materials and Methods

Materials

CYP3A4-transfected Caco-2 cells were obtained from Gentest (Woburn, MA). Dubach's Eagle's Medium containing 8.5 g/L glucose, 25 mM HEPES and 2.2 g/L $NaHCO_3$ and non-essential amino acids (NEAA), custom made, and Hanks' BSS buffer solution (0.4 g/L KCl, 0.06 g/L KH_2PO_4, 8.0 g/L NaCl, 1 g/L glucose, 0.09 g/L $Na_2HPO_4.7H_20$, 0.35 g/L $NaHCO_3$, without Phenol red, 0.1 μm sterile filtered) with calcium and magnesium salts and Penicillin G 10,000 units/mL plus Streptomycin SO_4 10,000 μg/mL solution were obtained from the UCSF Cell Culture Facility (San Francisco, CA). Fetal bovine serum (FBS) was obtained from HyClone Laboratories (Logan, UT, USA), and Hygromycin B was purchased from GIBCO BRL (Gaithersburg, MD, USA). Falcon polyethylene terephtalate (PET) 0.4 μm cell culture inserts (diameter 4.2 cm^2) were obtained from Becton Dickinson Labware, Franklin Lakes, NJ, USA and Costar 6-well plates were obtained from Fischer Scientific (Santa Clara, CA, USA). Sodium butyrate

(NaB) and O-tetradecanoylphorbol 13-acetate (TPA) were purchased from Sigma (St. Louis, MO).

Cell culture growth conditions

Cell culture medium was prepared by adding 10% fetal bovine serum (inactivated at 55°C), 1% Streptomycin/Penicillin solution and 0.2% Hygromycin solution to the custom made medium. After filtration (0.2 μm filter), this medium was heated to 37°C. CYP3A4-transfected Caco-2 cells (passages 4-5) were seeded at a density of 2.5 x 10^5 cells/insert and grown to confluence for 13-14 days. Twenty-four hours prior to the experiment, the cell culture media was replaced with growth media (cell culture media containing either 4 mM NaB and 100 nM TPA) to induce protein expression. Plates were incubated at 37°C, 5% CO_2 for 24 hours.

Transport and metabolism study in cell monolayers

Cell monolayers were preincubated in transport buffer (Hanks' buffer solution containing 25 mM HEPES and 1% FBS, adjusted to pH 7.4) for 30 minutes at 37°C. Transepithelial electrical resistance (TEER) values were measured across the monolayers using the Millipore Millicell system (Bedford, MA) equipped with chopstick electrodes. The study was initiated by preparing the receiver solution (Hanks' transport buffer plus 1% DMSO). The average TEER values obtained were 163.3 ± 20.9 ohm.cm2 (n=18). In each study, four six-well plates were divided as follows: 1) Control group, 2) GG 918 (Pg-p inhibitor), 3) Cyclosporine A (both Pg-p and CYP3A inhibitor). GG 918 and Cyclosporine A were added to the final concentration of 200 nM and 10 μM, respectively. The donor solutions were prepared by adding CLR to a final concentration of 5.0 μg/mL. After 30 minutes of incubation, the transport buffer was removed by aspiration and the donor and receiver solutions were added to the final volume of 1.5 mL in the apical side and 2.5 mL in the basolateral side.

An aliquot of 100 μL was taken from the receiver side after 30 minutes, 1 h and 2 h of incubation, and this volume was immediately replaced with fresh receiver solution to

preserve the original volume. The sample was then transferred to HPLC vials for LC-MS-MS analysis.

LC/MS Analysis

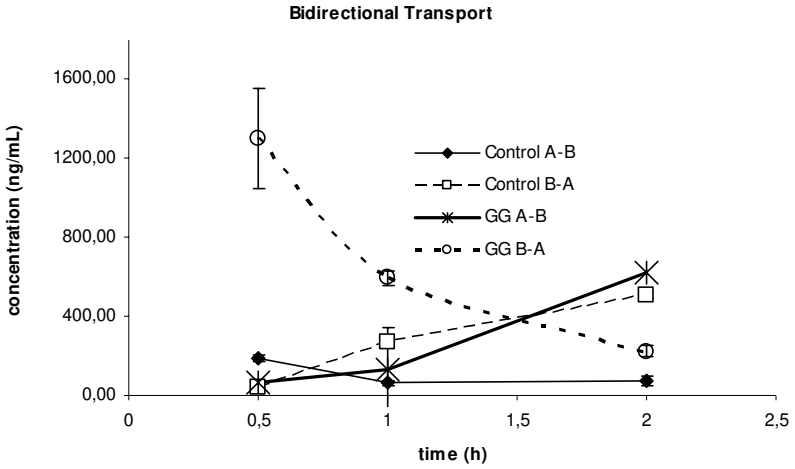
All transported parent drug and metabolite samples were analyzed by HPLC/electrospray-MS in combination with an on-line column switching extraction step using an HP1100 LC connected to a 5989B mass spectrometer through a 59987A electrospray interface (all Agilent Technologies). Separations were performed using a Agilent Zorbax, Eclipse-XDB-C8 analytical column, with 3.5 μm 50 mm x 4.6 mm i.d., at room temperature. The mobile phase was 30% acetonitrile, 70% water added by 0.1 % formic acid 10 mM (water). The flow rate was 0.9 mL/min and the total run time was 3.4 minutes. The mass spectrometer was run in the positive mode (ES+) and set for multiple reaction monitoring (MRN). The full-scan single-mass spectrum and the daughter ion-mass spectrum for CLR and 14-OH were (m/z) 749 > 590.6 and 765 > 158, respectively. The retention times for both compounds were 2.7 and 1.08 minutes, respectively for CLR and 14-OH.

Statistical analysis

CLR bidirectional transport across CYP3A4-Caco-2 cells monolayers was analysed by Friedman test ($p=0.05$).

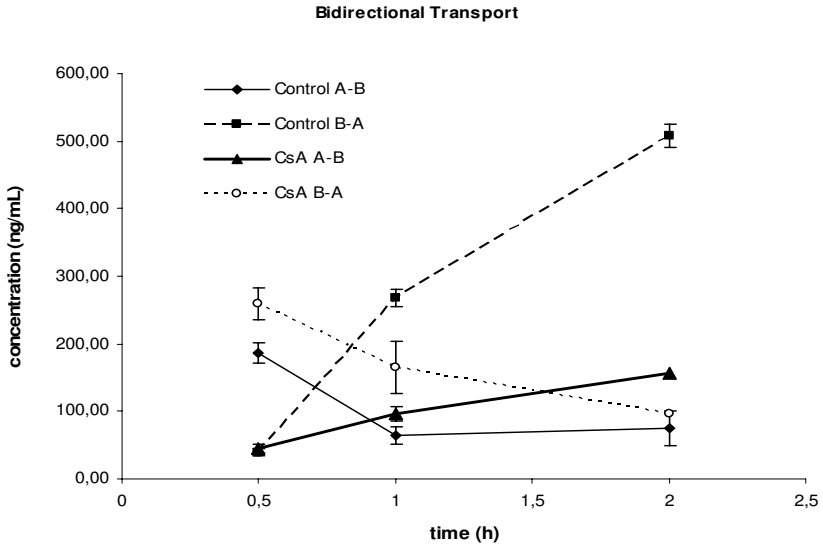
Results

Clarithromycin transport pattern was tested across CYP3A4 Caco-2 cells monolayers. CLR transport in the basolateral (B) to apical (A) direction was higher then the A to B direction, showing the P-gp activity in the efflux of CLR in the control group. In the presence of the P-gp inhibitor (GG918, 200 nM) a significant decrease in the B to A CLR transport was observed ($p < 0.05$). The B to A transport was higher in the first time point (30 minutes) and decreased after 1 and 2 h, showing concentrations similar to the control. Graphic 1 shows the CLR bidirectional transport across CYP3A4 Caco-2 cells in the presence of the P-gp inhibitor, GG918 200 nM.



Graphic 1. CLR bidirectional transport across CYP3A4 Caco-2 cells in the presence of the P-gp inhibitor, GG918 200 nM.

In the presence of the P-gp and CYP3A4 inhibitor (CsA 10 µM) a significant decrease ($p < 0.05$) in the B to A CLR transport was observed. The B to A transport was higher in the first time point (30 minutes) and decreased after 1 and 2 h. These data are demonstrated at Graphic 2.



Graphic 2. CLR bidirectional transport across CYP3A4 Caco-2 cells in the presence of the P-gp and CYP3A4 inhibitor (CsA 10 µM).

The *in vitro* formation of 14-OH and its bidirectional transport across CYP3A4 Caco-2 cells in the presence of CYP3A4 inhibitors, CsA 10 μM and the control group, are presented at Figure 1.

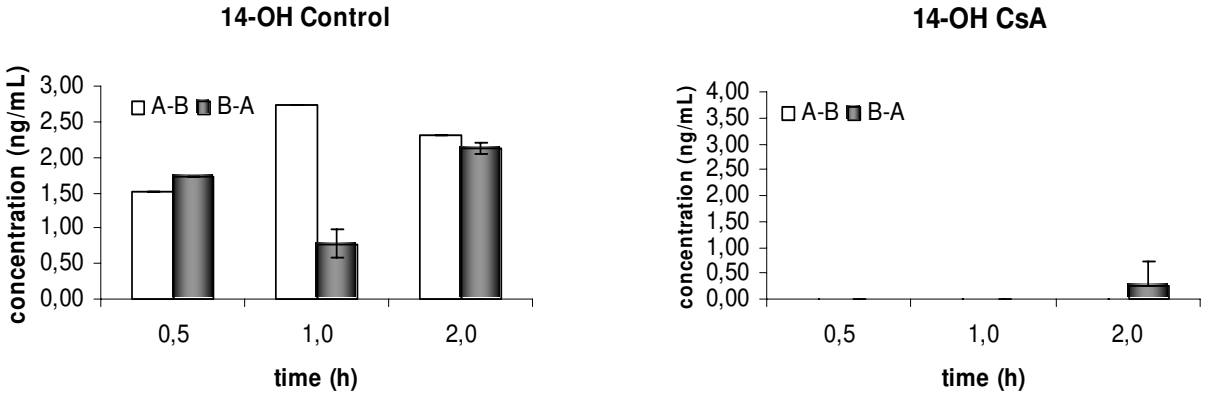


Figure 1. 14-OH-clarithromycin bidirectional transport across CYPA4 Caco-2 cells in the presence of CsA 10 μM.

Discussion

CLR concentrations have been determined by different methods. Chu *et al.*, (1991) described a HPLC method with electrochemical detection to quantify CLR plasmatic concentrations, further used in many other studies (CHU *et al*., 1992a,b,c, CHU *et al*., 1993a,b). Sastre Torano & Guchelaar (1998) described a HPLC method for the determination of the CLR using a fluorescence detector that showed linearity, accuracy and precision, but high LOQ (0.19 μg/mL) and total run time of 15.7 minutes.

The LC-MS-MS analysis performed by Lerner *et al.* (2000) obtained a LOQ of 5 ng/ml for CLR. In the present study, the analytical method described improved the sensitivity of the analytical assay, since low concentrations of studied compounds were detected (limits of quantification of 0.5 ng/mL and 0.2 ng/mL, respectively for CLR and 14-OH), with a reduced run time.

The CYP3A4-transfected Caco-2 cells model was used in this study to evaluate the importance of various biochemical factors in limiting drug absorption. Although transport studies can be performed in normal Caco-2 cells to determine if a drug is a substrate for

efflux transport, and intestinal microsomes can be used to calculate the expected intestinal intrinsic clearance, the relative importance of each of these individual factors in limiting overall drug absorption cannot easily be anticipated unless they are studied in combination (Cummins *et al.*, 2002).

The role of P-gp in the intestinal transport of drugs has been increasingly studied (Benet *et al.*, 1996a,b; Hall *et al.*, 1999; Cummins *et al.*, 2001). Previous reports demonstrated that CLR decreased the renal digoxin clearance and also inhibited the *in vitro* transcellular transport of digoxin from the basolateral to the apical (B to A) side in a concentration-dependent manner (Wakasugi *et al.*, 1998). This information suggested the inhibitory effect of CLR on the transport of other drugs, but its own absorption pattern is still not elucidated.

In the present study, CLR alone demonstrated that its transport in the B to A direction was about 5-fold higher than the A to B direction (absorptive direction) transport, showing the P-gp activity in the efflux of CLR in the control group. When a P-gp inhibitor (GG918, 200 nM) was used, the interaction with CLR resulted in a marked decrease in the B to A transport. However, the B to A transport is much higher in the first time point (30 minutes) and decreases after 1 and 2 h, showing concentrations similar to the control. In a typical inhibition curve, the demonstration of a marked P-gp effect should show concentrations much lower than the control group, even in the first time points (Cummins *et al.*, 2001). The results obtained in this study could possibly demonstrate a time-dependent P-gp inhibition, especially when CLR and GG918 are simultaneously added to the cell monolayers, as previously observed *in vivo* (Tanaka *et al.*, 2003).

Clinical and *in vitro* drug interactions have demonstrated that CLR inhibits CYP3A4 activity (Furuta *et al.*, 1999; Mayhew *et al.*, 2000; Sapone *et al.*, 2003; Mensa *et al.*, 2003; Yan *et al.*, 2002; Williams *et al.*, 2002; Moore *et al.*, 2002; Prieto 2001; Pea & Furlanut, 2001; Niemi *et al.*, 2001). Our data showed that the CsA inhibition on P-gp and CYP3A4 resulted in a significant decrease in the B to A transport after CsA exposition, 2-fold to 5-fold after 1 and 2 hours, respectively. On the other hand, CLR the A to B transport increased about 2-fold after 1 and 2 hours due to the CYP3A4 inhibition.

T The CLR active metabolite (14-OH), formed after incubation of CLR in CYP3A-tranfected Caco-2 cells, was mostly transported in the A to B direction, demonstrating the passive absorption of CLR and its intestinal first pass-extraction by CYP3A4. The results also indicate that both CYP3A4 and P-gp were not completely inhibited by CsA activity, in the concentrations used in the present study, in agreement with the 14-OH formation obtained after 2 hours of CsA inhibition (Figure 1).

The data obtained with CLR from CYP3A4-transfected Caco-2 cells suggests that multiple transporters could be involved in the disposition of this drug, since the effect of P-gp on metabolism was not similar to other drugs considered as typical P-gp substrates. Also, this effect appears to be time-dependent, and further studies with different time points and different concentrations of both drug and inhibitors are needed to completely elucidate the role of intestinal intracellular enzymes and transporters as absorption barriers to clarithromycin.

Acknowledgment

This study was supported by a grant from NIH (National Institute of Health, USA) to Dr. Leslie Z. Benet. The author Ana Paula Del Bortolo Ruenis was supported at the Department of Biopharmaceutical Sciences at UCSF, San Francisco, CA, USA, by a scholarship provided by CAPES.

References

1. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I and Gottesman MM (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu Rev Pharmacol Toxicol* **39**:361-398.
2. Benet LZ, Kroetz DL and Sheiner LB (1996a) The dynamics of drug absorption, distribution, and elimination., in *Goodman & Gilman's The Pharmacological Basis of Therapeutics* (Hardman JG, Limbird LE, Molinoff PB, Ruddon RW and Gilman AG eds) pp 3-28, McGraw-Hill, New York.

3. Benet LZ, Wu CY, Hebert MF and Wacher VJ (1996b) Intestinal drug metabolism and antitransport processes: a potential paradigm shift in oral drug delivery. *J Control Release* **39**:139-143.
4. Brophy DF, Israel DS, Pastor A, Gillotin C, Chittick GE, Symonds WT, Lou Y, Sadler BM and Polk RE (2000) Pharmacokinetic interaction between amprenavir and clarithromycin in healthy male volunteers. *Antimicrob Agents Chemother* **44**:978-984.
5. Chu S, Park Y, Locke C, Wilson DS and Cavanaugh JC (1992c) Drug-food interaction potential of clarithromycin, a new macrolide antimicrobial. *J Clin Pharmacol* **32**:32-36.
6. Chu S, Wilson DS, Deaton RL, Mackenthun AV, Eason CN and Cavanaugh JH (1993b) Single- and multiple-dose pharmacokinetics of clarithromycin, a new macrolide antimicrobial. *J Clin Pharmacol* **33**:719-726.
7. Chu SY, Deaton R and Cavanaugh J (1992a) Absolute bioavailability of clarithromycin after oral administration in humans. *Antimicrob Agents Chemother* **36**:1147-1150.
8. Chu SY, Granneman GR, Pichotta PJ, Decourt JP, Girault J and Fourtillan JB (1993a) Effect of moderate or severe hepatic impairment on clarithromycin pharmacokinetics. *J Clin Pharmacol* **33**:480-485.
9. Chu SY, Wilson DS, Guay DR and Craft C (1992b). Clarithromycin pharmacokinetics in healthy young and elderly volunteers. *J Clin Pharmacol* **32**:1045-1049.
10. Chu SY, Sennello LT and Sonders RC (1991) Simultaneous determination of clarithromycin and 14(R)-hydroxyclarithromycin in plasma and urine using high-performance liquid chromatography with electrochemical detection. *J Chromatogr* **571**(1-2):199-208.
11. Cummins CL, Mangravite LM and Benet LZ (2001) Characterizing the expression of CYP3A4 and efflux transporters (P-gp, MRP1, and MRP2) in CYP3A4-transfected Caco-2 cells after induction with sodium butyrate and the phorbol ester 12-O-tetradecanoylphorbol-13- acetate. *Pharm Res* **18**:1102-1109.
12. Cummins CL, Jacobsen W and Benet LZ (2002) Unmasking the dynamic interplay between intestinal Pglycoprotein and CYP3A4. *J Pharmacol Exp Ther* **300**:1036-1045.

13. Fraschini F. (1990) Clinical efficacy and tolerance of two new macrolides, clarithromycin and josamycin, in the treatment of patients with acute exacerbations of chronic bronchitis. J. Int. Med. Res. **18**: 171-176.
14. Furuta T, Ohashi K, Kobayashi K, Iida I, Yoshida H, Shirai N, Takashima M, Kosuge K, Hanai H, Chiba K, Ishizaki T and Kaneko E (1999) Effects of clarithromycin on the metabolism of omeprazole in relation to CYP2C19 genotype status in humans. *Clin Pharmacol Ther* **66**:265-74.
15. Hall SD, Thummel KE, Watkins PB, Lown KS, Benet L.Z, Paine MF, Mayo RR, Turgeon DK, Bailey DG, Fontana RJ and Wrighton SA (1999). Molecular and physical mechanisms of first pass extraction. *Drug Metab Dispos* **27**:161-166.
16. Jones RN, Erwin ME and Barrett MS (1990) *In vitro* activity of clarithromycin (TE-031, A-67268) and 14-0H clarithromycin alone and in combination against *Legionella* species. *Eur. J. Clin. Microb. Infect.Dis*. **9**: 846-848.
17. Lerner FE, Caliendo G, Santagada V, Santana GSM, Moraes MEA, and De Nucci G (2000) Clarithromycin bioequivalence study of two oral formulations in healthy human volunteers. *Int J Clin Pharmacol Ther* **38**:345-354.
18. Mayhew BS, Jones DR, Hall SD (2000) An *in vitro* model for predicting *in vivo* inhibition of cytochrome P450 3A4 by metabolic intermediate complex formation. *Drug Metab Dispos* **28**:1031-1037.
19. Mensa J, Garcia-Vazquez E and Vila J (2003) Macrolides, ketolides and streptogramins. *Enferm Infecc Microbiol Clin* **21**:200-207.
20. Moore KH, Leese PT, McNeal S, Gray P, O'Quinn S, Bye C and Sale M (2002) The pharmacokinetics of sumatriptan when administered with clarithromycin in healthy volunteers. *Clin Ther* **24**:583-594.
21. Niemi M, Neuvonen PJ and Kivisto KT (2001) The cytochrome P4503A4 inhibitor clarithromycin increases the plasma concentrations and effects of repaglinide. *Clin Pharmacol Ther* **70**:58-65.
22. Pea F and Furlanut M (2001) Pharmacokinetic aspects of treating infections in the intensive care unit: focus on drug interactions. *Clin Pharmacokinet* **40**:833-68.
23. Prieto JC (2001) Safety profile of statins. *Rev Med Chil* **129**:1237-1240.

24. Sapone A, Vaira D, Trespidi S, Perna F, Gatta L, Tampieri A, Ricci C, Cantelli-Forti G, Miglioli M, Biagi GL and Paolini M (2003) The clinical role of cytochrome p450 genotypes in *Helicobacter pylori* management. *Am J Gastroenterol* **98**:1010-1015.
25. Sastre Torano J and Guchelaar HJ (1998) Quantitative determination of the macrolide antibiotics erythromycin, roxithromycin, azithromycin and clarithromycin in human serum by high-performance liquid chromatography using pre-column derivatization with 9-fluorenylmethyloxycarbonyl chloride and fluorescence detection. *J Chromatogr B Biomed Sci Appl* **720**(1-2):89-97.
26. Tanaka H, Matsumoto K, Ueno K, Kodama M, Yoneda K, Katayama Y and Miyatake K (2003) Effect of clarithromycin on steady-state digoxin concentrations. *Ann Pharmacother* **37**:178-181.
27. Wakasugi H, Yano I, Ito T, Hashida T, Futami T, Nohara R, Sasayama S and Inui K (1998) Effect of clarithromycin on renal excretion of digoxin: interaction with P-glycoprotein. *Clin Pharmacol Ther* 64:123-128.
28. Wang L, Kitaichi K, Hui CS, Takagi K, Takagi K, Sakai M, Yokogawa K, Miyamoto KI and Hasegawa T (2000) Reversal of anticancer drug resistance by macrolide antibiotics *in vitro* and *in vivo*. *Clin Exp Pharmacol Physiol* 27:587-593.
29. Williams JA, Ring BJ, Cantrell VE, Jones DR, Eckstein J, Ruterbories K, Hamman MA, Hall SD and Wrighton SA (2002) Comparative metabolic capabilities of CYP3A4, CYP3A5, and CYP3A7. *Drug Metab Dispos* **30**:883-891.
30. Yan Z, Rafferty B, Caldwell GW and Masucci JA (2002) Rapidly distinguishing reversible and irreversible CYP450 inhibitors by using fluorometric kinetic analyses. *Eur J Drug Metab Pharmacokinet* **27**:281-287.

6 DISCUSSÃO

A adoção de uma diretriz para medicamentos genéricos, envolvendo a produção, a garantia de qualidade, a prescrição, a dispensação e o uso dos mesmos, é parte fundamental de uma orientação para promoção do uso racional de medicamentos no Brasil, sendo uma das principais diretrizes preconizadas pela Organização Mundial de Saúde – OMS (Brasil, 1999a).

No Brasil, a partir da instituição do medicamento genérico através da Lei n° 9.787 de 10/02/1999 (Brasil, 1999b), a ANVISA/MS tem publicado guias para a elaboração e condução de estudos de biodisponibilidade e bioequivalência de medicamentos (Brasil, 2000; Brasil, 2001; Brasil, 2002a,b,c,d).

Os protocolos de bioequivalência são, em geral ensaios do tipo aberto, aleatório, cruzado, em dois períodos, cujas coletas devem contemplar o período correspondente a no mínimo três a cinco meias-vidas de eliminação do fármaco, com período mínimo de intervalo (*washout*) entre os dois períodos de cinco a sete meias-vidas de eliminação. O ensaio de bioequivalência deve ser conduzido usando-se método analítico exato, preciso e reprodutível para assegurar a obtenção dos parâmetros farmacocinéticos necessários à determinação de bioequivalência (Brasil, 2002).

Recentemente, a ANVISA/MS (Brasil, 2003a,b,c,d,e) publicou novos guias para a elaboração e execução de estudos de bioequivalência, contemplando as etapas clínica, analítica e estatística desses ensaios. Dentre as principais alterações com relação à legislação anterior, destaca-se a possibilidade de utilização do parâmetro área sob a curva truncada (ASC_{0-72}), através da utilização de um cronograma de coletas alternativo, de zero a 72 horas após a administração de fármaco com meia-vida de eliminação superior a 24 horas (Brasil, 2003b).

O FDA recomenda que nos estudos de bioequivalência com fármacos de meia-vida elevada seja preferencialmente utilizado o desenho cruzado, em dose única ou, caso isto não seja possível, utilizar desenho paralelo (Marzo, 1999; United States, 2003). Em ambos os casos, é recomendado que o período de coleta das amostras seja adequado para

assegurar completo trânsito do fármaco no trato gastrointestinal (aproximadamente 2 ou 3 dias), bem como sua absorção.

Para os fármacos com baixa variabilidade intraindividual com relação à distribuição e eliminação, a área sob a curva truncada em 72 horas (ASC_{0-72}) pode ser usada em substituição a ASC_{0-t} e $ASC_{0-\infty}$. No entanto, para fármacos que apresentem grande variabilidade intraindividual com relação aos parâmetros de distribuição e eliminação, a utilização de ASC_{0-72} deve ser feita somente após consulta ao FDA (United States, 2003). Para estes casos, de maneira geral, a determinação precisa de ASC_{0-t}, ASC_{0-inf} e C_{max} requer um número elevado de coletas de sangue, aumentando o tempo e o custo do estudo (Mahmood & Mahayni, 1999).

Tem sido discutida a utilização de uma estratégia de coleta de amostras limitadas (LSS, do *inglês limited-sampling strategy* ou *limited sampling method*, LSM), a fim de reduzir o número de coletas de sangue sem comprometer a estimativa dos parâmetros de absorção ASC_{0-t}, ASC_{0-inf} e C_{max} (Mahmood & Mahayni, 1999; Suarez–Kurtz *et al.*, 1999).

Mahmood & Mahayni (1999) avaliaram amostras de sangue de 15 voluntários saudáveis após a administração de um fármaco de $T_{1/2}$ elevada (> 35 h) ou de um fármaco de $T_{1/2}$ curta (= 12 h), em estudo de bioequivalência com desenho cruzado, com dois períodos. A relação entre as concentrações plasmáticas obtidas e os parâmetros ASC_{0-t} e C_{max} foi avaliada através de análise de regressão linear múltipla. Neste modelo, foram escolhidos cinco pontos de coleta cujas concentrações exibiram melhor correlação com os parâmetros de absorção (r > 0,9), cujos resultados demonstraram que a diferença entre os parâmetros observados com maior número de coleta e aqueles estimados por LSM foram menores que 5%, assegurando assim a aplicabilidade deste sistema a estudos de bioequivalência (Mahmood & Mahayni, 1999).

Modelo semelhante foi descrito para avaliar a LSS aplicada ao estudo de bioequivalência do itraconazol, um fármaco de alta variabilidade interindividual, alta taxa de distribuição tecidual, $T_{1/2}$ de 24 h, e caracterizado pela formação *in vivo* de cerca de 30

metabólitos, dos quais o hidróxi-itraconazol demonstra atividade antifúngica (Suarez–Kurtz *et al.*, 1999). Foi demonstrado que um modelo baseado em três coletas de sangue poderia garantir a obtenção de parâmetros ASC_{0-t} e C_{max} ($R^2 > 0,98$).

Na literatura científica, são escassas as informações relativas às variabilidades intra e inter individuais que permitam estimar ASC_{0-72} (truncada) e LSS para a elaboração de protocolos de biodisponibilidade/bioequivalência de medicamentos, levando à necessidade de realização de estudo piloto. Nos Estudos 1 e 2, os parâmetros farmacocinéticos obtidos com a carbamazepina e claritromicina podem servir de amostra de conveniência para a estimativa de ASC_{0-72} (truncada) e LSS, de forma semelhante a modelos previamente descritos (Mahmood & Mahayni, 1999; Suarez–Kurtz *et al.*, 1999).

A posterior publicação destes resultados, bem como de outros estudos de bioequivalência pela comunidade científica pode auxiliar os pesquisadores no delineamento de protocolos mais adequados, com menor exposição do voluntário a um procedimento invasivo como a coleta de sangue, e custo reduzido.

Os estudos de fase I, bem como os estudos de biodisponibilidade/bioequivalência podem ser aplicados à estimativa da eficácia de fármacos antimicrobianos. A investigação da influência dos parâmetros farmacocinéticos sobre o efeito dos antimicrobianos *in vivo* e sua aplicação na utilização racional de antimicrobianos têm sido amplamente discutidas, uma vez que a manutenção de concentrações séricas e teciduais em função do tempo é essencial para atingir a eficácia terapêutica (Turnidge, 1998; Li, 2000; Amsden, 2001; Van Bambeke & Tulkens, 2001; Fridt-Moller, 2002; Toultain *et al*., 2002; Bonapace *et al*., 2002). Estes autores apresentam algumas opiniões divergentes quanto ao melhor método preditivo de eficácia antimicrobiana *in vivo*.

No caso dos macrolídeos, a determinação isolada de %T>CIM, como nos β-lactâmicos, ou C_{max}/CIM, como nos aminoglicosídeos, é insuficiente para avaliar de forma preditiva sua atividade clínica. Ao contrário, tem sido relatado que as determinações tanto da %T>CIM quanto da ASC_{24h}/CIM podem influenciar a atividade clínica dos macrolídeos considerados de geração avançada, como a azitromicina e a claritromicina (Amsden, 2001;

Van Bambeke & Tulkens, 2001).

Sob este aspecto, a determinação dos parâmetros T>CIM, %T>CIM e ASC_{0-24h}/CIM através da utilização de dados farmacocinéticos obtidos em estudo de bioequivalência, juntamente com a eficácia *in vivo* da claritromicina na redução da microbiota oral e nasal de voluntários saudáveis demonstrou ser um modelo capaz de correlacionar estes diversos fatores.

No entanto, a claritromicina possui características diferentes de outros macrolídeos, que não têm sido consideradas em conjunto na determinação de seus parâmetros PK/PD. Este fármaco, ao contrário da azitromicina, não possui um efeito pós-antibiótico marcante, mesmo considerando-se os efeitos tanto do composto original quanto de seu metabólito, respectivamente 1,5 h e 3 h (Jung *et al.*, 2002). No entanto, seu metabólito ativo, 14-OH, atinge altas concentrações plasmáticas, até cerca de 40 a 50% do composto original (LERNER *et al.*, 2000).

Com este trabalho, portanto, sugerimos a necessidade de incorporar os dados farmacocinéticos referentes ao metabólito 14-OH na determinação dos parâmetros PK/PD preditivos de eficácia antimicrobiana não só da claritromicina, mas também de outros fármacos que possuam metabólitos ativos.

A investigação do comportamento farmacocinético *in vitro* da claritromicina em modelo de células CYP3A4-Caco-2 foi conduzida de acordo com o modelo delineado e validado por Cummins *et al.* (2001). Este modelo, cujas células superexpressam a P-gp e a CYP3A4, tem sido utilizado para a investigação de diferentes fármacos, como o midazolam (Wacher *et al.*, 2001), o albendazol (Kobayashi *et al.*, 2001), a ciclosporina (Wacher *et al.*, 2001), compostos polifenólicos de atividade anticarcinogênica presentes no chá verde (Jodoin *et al.*, 2002), entre outros.

Entretanto, além da P-gp, já foram identificados outros transportadores de efluxo nas células intestinais, incluindo as proteínas de resistência ao câncer de mama (BCRP, do inglês *breast cancer resistance protein*) proteínas associadas de multi-resistência (MRPs, do inglês *multidrug resistance associated proteins*), os transportadores do ácido carboxílico (MCT1, do inglês *monocarboxylic acid transporter*) e transportadores de nuclosídeo (ENT1, do inglês *equilibrative nucleoside transporter*).

A MRP1, a MRP3 e a MRP5 localizam-se na membrana basolateral dos enterócitos, enquanto que a MRP2 possui localização apical (Borst *et al.*, 2000). Em geral, estas proteínas transportam compostos conjugados como os glicuronídeos, mas tem sido relatado que substratos não conjugados como inibidores de protease e a pravastatina também são substratos para as MRPs (Evers *et al.*, 1996; Sheffer *et al.*, 2000; Taipalensuu *et al.*, 2001).

A BCRP é também um membro da superfamília de transportadores ABC (como a P-gp), localizada na região apical do enterócito e responsável pelo efluxo de fármacos antineoplásicos como o topotecan e a minoxantrona, limitando assim sua absorção intestinal (Jonker *et al.*, 2000). Os transportadores MCT1 e ENT1 estão presentes tanto na membrana apical quanto na basolateral, promovendo a captação (*uptake*) de seus substratos do lúmen para o interior do enterócito e seu posterior efluxo na corrente sangüínea, favorecendo assim a absorção de fármacos contra um gradiente de concentração (TAMAI *et al.*, 1999; PENNYCOOK *et al.*, 2001).

No estudo 4, foi demonstrada atividade da CYP3A4 sobre a extração intestinal da claritromicina, e sua interação com a P-gp no efluxo deste fármaco. Entretanto, não foi observada uma curva típica de transporte bidirecional para um forte substrato de P-gp e CYP3A4, indicando que a claritromicina pode estar sofrendo a ação de outros transportadores. Assim, sugere-se a necessidade de realizar estudos *in vitro* com linhagens celulares que superexpressem outros transportadores de efluxo, para a completa elucidação dos fatores que interferem na absorção intestinal da claritromicina e, conseqüentemente, na sua biodisponibilidade.

Os modelos farmacocinéticos *in vitro* e *in vivo* apresentados neste trabalho são adequados para avaliar aspectos importantes da disposição do fármaco no organismo, sendo aplicáveis tanto nos estágios iniciais de desenvolvimento de fármacos como na melhor compreensão das propriedades farmacocinéticas daqueles já registados e comercializados.

Este trabalho de tese faz parte de um processo de implantação de uma nova linha de pesquisa inserida no Programa de Pós-Graduação em Odontologia (PPG-O) da FOP/UNICAMP, possibilitando ao grupo de pesquisadores (orientador, orientada, o Prof. Dr. Francisco Carlos Groppo, bem como o Prof. Luiz Madaleno Franco da UNIMEP) habilitar, junto a ANVISA/MS, um Centro de Estudos de Biodisponibilidade e

Bioequivalência de Medicamentos (CEB), em conjunto com a Bioagri Laboratórios Ltda. e o Hospital dos Fornecedores de Cana de Piracicaba. Assim sendo, este processo contribuiu significativamente para a formação científica e profissional de pesquisadores e alunos de Mestrado e Doutorado desta Área.

7 CONCLUSÃO

Os modelos de farmacocinética *in vitro* e *in vivo* apresentados neste trabalho mostraram-se adequados para avaliar aspectos importantes da disposição do fármaco no organismo, sendo aplicáveis tanto nos estágios iniciais de desenvolvimento de fármacos como na melhor compreensão das propriedades farmacocinéticas de medicamentos já registrados e comercializados.

Conclusões específicas:

- o medicamento teste de carbamazepina foi considerado bioequivalente ao medicamento de referência, baseando-se na velocidade e extensão de absorção, podendo assim, serem intercambiáveis (estudo 1);

- o medicamento teste de claritromicina foi considerado bioequivalente ao medicamento de referência, baseando-se na velocidade e extensão de absorção, podendo assim, serem intercambiáveis (estudo 2);

- a claritromicina mostrou-se eficaz na redução da microbiota oral e nasal de voluntários saudáveis e a utilização do modelo apresentado promoveu uma melhor estimativa *in vivo* da eficácia da claritromicina, quando comparado aos modelos *in vitro* tradicionais (estudo 3);

- a claritromicina demonstrou ser um substrato para os efeitos de primeira-passagem intestinal mediados pela P-gp e CYP3A4, no modelo *in vitro* com células CYP3A4-Caco-2, e novos estudos envolvendo diferentes transportadores são necessários à completa elucidação deste mecanismo (estudo 4).

REFERÊNCIAS

01. Amsden GW. Advanced generation macrolides: tissue-directed antibiotics. ***Int J Antimicrob Agents***. 2001; 18 Suppl 1: S11-5.

02. Artursson P, Borchardt RT. Intestinal drug absorption and metabolism in cell culture: Caco-2 and beyond**.** ***Pharm Res***. 1997 Dec; 14(12): 1655-8.

03. Artursson P, Karlsson, J. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal ephitelial (Caco-2) cells. ***Biochem Biophys Res Commun***. 1991 Mar 29; 175(3): 880-5.

04. Benet LZ, Cummins CL. The drug efflux-metabolism alliance: biochemical aspects. ***Adv Drug Deliv Rev***. 2001 Oct 1; 50 Suppl 1: S3-11.

05. Bonapace CR, Friedrich LV, Bosso JA, White, RL. Determination of antibiotic effect in an in vitro pharmacodynamic model: comparison with an established animal model of infection. ***Antimicrob Agents Chemother***. 2002 Nov; 46(11): 3574-9.

06. Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. ***J Natl Cancer Inst.*** 2000 Aug 16; 92(16): 1295-302.

07. Braga RMC. Otimização e validação analítica do tamoxifeno em plasma, avaliação da liodisponibilidade e biodisponibilidade de especialidades farmacêuticas sólidas à base do citrato de tamoxifeno comercializadas no Brasil. [tese] Recife: Faculdade de Ciências Farmacêuticas da Universidade Federal de Pernambuco, 2000.

08. Brasil. Lei n° 9.787, de 10 de fevereiro de 1999. Diário Oficial da República Federativa do Brasil, Brasília, 10 de fevereiro de 1999b.

09. Brasil. Resolução n° 391, de 9 de agosto de 1999. Regulamento técnico para medicamentos genéricos - 1/1999. ***Diário Oficial da República Federativa do Brasil***, Brasília, 10 de agosto de 1999.

* De acordo com a norma da UNICAMP/FOP, baseada no modelo de Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

10. Brasil. Resolução-RDC n° 10, de 2 de janeiro de 2001. Regulamento técnico para medicamentos genéricos - 1/2001. ***Diário Oficial da República Federativa do Brasil***, Brasília, 9 de janeiro de 2001.

11. Brasil. Resolução-RDC n° 135, de 29 de maio de 2003. Regulamento técnico para medicamentos genéricos - 1/2003. ***Diário Oficial da República Federativa do Brasil***, Brasília, Republicada em 12 de agosto de 2003a.

12. Brasil. Resolução-RDC n° 84, de 19 de março de 2002. Regulamento técnico para medicamentos genéricos - 1/2002. ***Diário Oficial da República Federativa do Brasil***, Brasília, 20 de março de 2002a.

13. Brasil. Resolução-RE n° 478, de 19 de março de 2002. Guia para provas de bioequivalência de medicamentos genéricos - 1/2002. ***Diário Oficial da República Federativa do Brasil***, Brasília, 20 de março de 2002b.

14. Brasil. Resolução-RE n° 479, de 19 de março de 2002. Guia para protocolo e relatório técnico de estudo de bioequivalência - 1/2002. ***Diário Oficial da República Federativa do Brasil***, Brasília, 20 de março de 2002c.

15. Brasil. Resolução-RE n° 484, de 19 de março de 2002. Guia para desenhos aplicáveis a estudo de bioequivalência - 1/2002. ***Diário Oficial da República Federativa do Brasil***, Brasília, 20 de março de 2002d.

16. Brasil. Resolução-RE n° 894, de 29 de maio de 2003. Guia para protocolo e relatório técnico de estudo de biodisponibilidade relativa/bioequivalência - 1/2003. ***Diário Oficial da República Federativa do Brasil***, Brasília, 2 de junho de 2003b.

17. Brasil. Resolução-RE n° 895, de 29 de maio de 2003. Guia para elaboração de relatório técnico de estudo bioequivalência - 1/2003. ***Diário Oficial da República Federativa do Brasil***, Brasília, 2 de junho de 2003c.

18. Brasil. Resolução-RE n° 898, de 29 de maio de 2003. Guia para planejamento e realização de etapa estatística de estudos de biodisponibilidade relativa/bioequivalência - 1/2003. ***Diário Oficial da República Federativa do Brasil***, Brasília, 2 de junho de 2003d.

19. Brasil. Resolução-RE n° 899, de 29 de maio de 2003. Guia para validação de métodos analíticos e bioanalíticos - 1/2003. ***Diário Oficial da República Federativa do Brasil***, Brasília, 2 de junho de 2003e.

20. Chaudhary PM, Mechetner EB, Roninson IB. Expression and activity of the multidrug resistance P-glycoprotein in human peripheral blood lymphocytes. ***Blood.*** 1992 Dec 1; 80(11): 2735-9.

21. Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR, *et al*. Mutidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. ***Proc Natl Acad Sci U S A***. 1989 Jan; 86(2): 695-8.

22. Credito KL, Ednie LM, Jacobs MR, Appelbaum PC. Activity of telithromycin (HMR 3647) against anaerobic bacteria compared to those of eight other agents by time-kill methodology. ***Antimicrob Agents Chemother***. 1999 Aug; 43(8): 2027-31.

23. Cummins CL, Mangravite LM, Benet LZ. Characterizing the expression of CYP3A4 and efflux transporters (P-gp, MRP1 and MRP2) in CYP3A4-transfected Caco-2 cells after induction with sodium butyrate and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate. ***Pharm Res***. 2001 Aug; 18(8): 1102-9.

24. Dajani AS, Bawdon RE, Berry MC. Prevention of bacterial endocarditis. Recommendations by the American Heart Association. ***J Am Med Ass.*** 1997 jun; 277(22): 1794-1801.

25. Domanski TL, Finta C, Halpert JR, Zaphiropoulos PG. cDNA cloning and initial characterization of CYP3A43, a novel human cytochrome P450. ***Mol Pharmacol***. 2001 Feb; 59(2): 386-92.

26. Dresser MJ, Leabman MK, Giacomini KM. Transporters involved in the elimination of drugs in the kidney: organic anion transporters and organic cation transporters. ***J Pharm Sci***. 2001 Apr; 90(4): 397-421.

27. Ebert SC, Craig WA. Pharmacodynamic properties of antibiotics: application to drug monitoring and dosage regimen design. ***Infect Control Hosp Epidemiol***. 1990 Jun; 11(6): 319-26.

28. Evers R, Zaman GJ, Van Deemter L, Jansen H, Calafat J, Oomen LC *et al*. Basolateral localization and export activity of the human multidrug-resistance associated protein in polarized pig kidney cells. ***J Clin Invest***. 1996 Mar 1; 97(5): 1211-8.

29. Frimodt-Moller N. How predictive is PK/PD for antibacterial agents? ***Int J Antimicrob Agents***. 2002 Apr; 19(4): 333-9.

30. Goldstein LJ. MDR1 gene expression in solid tumors. ***Eur J Cancer***. 1996 Jun; 32A(6): 1039-50.

31. Gutmann H, Fricker G, Torok M, Michael S, Beglinger C, Drewe J. Evidence for different ABC-transporters in Caco-2 cells modulating drug uptake. ***Pharm Res***. 1999 Mar; 16(3): 402-7.

32. Jodoin J, Demeule M, Beliveau R. Inhibition of the multidrug resistance P-glycoprotein activity by green tea polyphenols. ***Biochim Biophys Acta***. 2002 Jan 30; 1542(1-3): 149-59.

33. Jonker JW, Smit JW, Brinkhuis RF, Maliepaard K, Beijnen JH, Schelens JH *et al*. Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. ***J Natl Cancer Inst***. 2000 Oct 18; 92(20): 1651-6.

34. Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. ***Biochim Biophys Acta***. 1976 Nov 11; 455(1): 152-62.

35. Jung H, Milán RC, Girard ME, León F, Montoya MA. Bioequivalence study of carbamazepine tablets: *in vitro*/*in vivo* correlation. ***Int J Pharm.*** 1997 152: 37-44.

36. Kobayashi M, Sada N, Sugawara M, Iseki K, Miyasaki K. Development of a new system for prediction of drug absorption into account drug dissolution and pH change in the gastrointestinal tract. ***Int J Pharm***. 2001 Jun 19; 221(1-2): 87-94.

37. Kolars JC, Lown KS, Schimiedlin-Ren P, Ghosh M, Fang C, Wrighton SA *et al*. CYP3A4 gene expression in human gut ephitelium. ***Pharmacogenetics***. 1994 Oct; 4(5): 247-59.

38. Lerner FE, Caliendo G, Santagada V, Santana GSM, Moraes MEA, De Nucci, G. Clarithromycin bioequivalence study of two oral formulations in healthy human volunteers. ***Int J Clin Pharmacol Ther.*** 2000 Jul; 38(7): 345-54.

39. Li RC. New pharmacodynamic parameters for antimicrobial agents. ***Int J Antimicrob Agents***. 2000 Feb; 13(4): 229-35.

40. Mahmood I, Mahayni H. A limited sampling approach in bioequivalence studies: application to long half-life drugs and replicate design studies. ***Int J Clin Pharmacol Ther***. 1999 Jun; 37(6): 275-81.

41. Marzo A. Open questions on bioequivalence: some problems and some solutions. ***Pharmacol Res***. 1999 Oct; 40(4): 357-68.

42. Mashford ML, Ryan PL, Thomson WA. Determination of carbamazepine in plasma. ***J Chromatogr***. 1974 Feb 13; 89(1): 11-5.

43. Meibohm B, Derendorf H. Pharmacokinetic/pharmacodynamic studies in drug product development. ***J Pharm Sci***. 2002 Jan; 91(1): 18-31.

44. Montgomery EH. Princípios e mecanismos da antibioticoterapia. *In*: Neidle EA, Yagiela JA. ***Farmacologia e terapêutica para dentistas***. 3.ed. Rio de Janeiro: Guanabara Koogan; 1991. p. 397-411.

45. Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ ***et al***. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. ***Pharmacogenetics***. 1996 Feb; 6(1): 1-42.

46. Oh DM, Han H, Amidon GL. Drug transport and targeting: intestinal transport. ***Membrane transporters as drug targets.*** Amidon, G.L., Sadee, W., Eds. New York, Kluwer Academic, p.59-88.

47. Paine MF, Khalighi M, Fisher JM, Shen DD, Kunze KL, Marsh CL *et al*. Characterization of interintestinal and intraintestinal variations in human CYP3A4-dependent metabolism. ***J Pharmacol Exp Ther***. 1997 Dec; 283(3): 1552-62.

48. Pennycooke M, Chaudary N, Shuralyova I, Zhang Y, Coe IR. Differential expression of human nucleoside transporters in normal and tumor tissue. ***Biochem Biophys Res Commun***. 2001 Jan 26; 280(3): 951-9.

49. Porta V. ***Avaliação da bioequivalência de formulações do mercado nacional contendo fluconazol.*** [tese] São Paulo - Faculdade de Ciências Farmacêuticas da Universidade de São Paulo, 1999.

50. Scheffer GL, Kool M, Heijn M, de Haas M, Pijnenborg AC, Wijnholds J *et al*. Specific detection of multidrug resistance proteins MRP1, MRP2, MRP3, MRP5 and MDR3 P-glycoprotein with a panel of monoclonal antibiotics. ***Cancer Res***. 2000 Sep 15; 60(18): 5269-77.

51. Schmiedlin-Ren P, Thummel KE, Fisher JM, Paine MF, Lown KS, Watkins PB. Expression of enzymatically active CYP3A4 by Caco-2 cells grown on extracellular matrix-coated permeable supports in the presence of 1alfa,25-dihydroxyvitamin D_3. ***Mol Pharmacol***. 1997 May; 51(5): 741-54.

52. Serra CHR. ***Avaliação biofarmacotécnica de comprimidos contendo cefalexina: cinética de dissolução e bioequivalência.*** [tese] - São Paulo: Faculdade de Ciências Farmacêuticas da Universidade de São Paulo, 1998.

53. Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP. Interindividual variations in human liver cytochrome P450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microssomes of 30 Japanese and 30 Caucasians. ***J Pharmacol Exp Ther***. 1994 Jul; 270(1): 414-23.

54. Spina E, Pisani F, Perucca **E**. Clinically significant pharmacokinetic drug interactions with carbamazepine. ***Clin Pharmacokinet***. 1996 Sep; 31(3): 198-214.

55. Storpirtis S. Biofarmácia e farmacocinética: ensino e pesquisa. ***Infarma***. 1993; 2(4): 19-20.

56. Storpirtis S, Consiglieri, VO. Biodisponibilidade e bioequivalência de medicamentos: aspectos fundamentais para o planejamento e execução de estudos**.** ***Rev. farm. bioquim. Univ. São Paulo***. Jul-dez 1995; 31(2): 63-70.

57. Suarez-Kurtz G, Bozza FA, Vicente FL, Ponte CG, Struchiner CJ. Limited-sampling strategy models for itraconazole and hydroxyl-itraconazole based on data from a bioequivalence study. ***Antimicrob Agents Chemother***. 1999 Jan; 43(1): 134-40.

58. Taipalensuu J, Tornblom H, Lindberg G, Einarsson C, Sjoqvist F, Melhus H *et al*. Correlation of gene expression of ten drug efflux proteins of the ATP-binding cassette transport family in normal human jejunum and in intestinal epithelial Caco-2 cell monolayers. ***J Pharmacol Exp Ther***. 2001 Oct; 299(1): 164-70.

59. Tamai I, Ohashi R, Nezu JI, Sai Y, Kobayashi D, Oku A *et al*. Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2. ***J Biol Chem***. 2000 Dec 22; 275(51): 40064-72.

60. Taubert KA & Dajani AS. Preventing bacterial endocarditis: American Heart Association guidelines. ***Am Fam Physician***. 1998 Feb 1; 57(3): 457-68.

61. Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC. Cellular localization of the multidrug- resistance gene product P-glycoprotein in normal human tissues. ***Proc Natl Acad Sci U S A***. 1987 Nov; 84(21): 7735-8.

62. Toutain PL, del Castillo JR, Bousquet-Melou A. The pharmacokinetic/pharmacodynamic approach to a racional regimen for antibiotics. ***Res Vet Sci***. 2002 Oct; 73(2): 105-14.

63. Turnidge JD. The pharmacodynamics of β-lactams. ***Clin Infect Dis***. 1998 Jul; 27(1): 10-22.

64. United States. Code of Federal Regulations. Title 21. Part 320 – Bioavailability and Bioequivalence Requirements. Washington: ***US Government Printing Office*** 1992 p.140-159.

65. United States. Guidance for Industry - bioavailability and bioequivalence studies for orally administered drug products – general considerations. Department of Health and Human Services. Food and Drug Administration, ***Center for Drug Evaluation and Research (CDER)*** 2003; 23p.

66. Van Bambeke F, Tulkens PM. Macrolides: pharmacokinetics and pharmacodynamics. ***Int J Antimicrob Agents***. 2001; 18 Suppl 1: S17-23.

67. Visalli MA, Jacobs MR, Appelbaum PC. Susceptibility of twenty penicillin-susceptible and –resistant pneumococci to levofloxacin, ciprofloxacin, ofloxacin, erythromycin, azithromycin and clarithromycin by MIC and time-kill. ***Diagn Microbiol Infect Dis***. 1997 Jul; 28(3): 131-7.

68. Wacher VJ, Salphati L, Benet LZ. Active secretion and enterocytic drug metabolism barriers to drug absorption. ***Adv Drug Deliv Rev***. 2001 Mar 1; 46(1-3): 89-102.

69. Wacher VJ, Silverman JA, Zhang Y, Benet LZ. Role of P-glycoprotein and cytochrome P450 3A in limiting drug absorption of peptides and peptidomimetics. ***J Pharm Sci***. 1998 Nov; 87(11): 1322-30.

70. Wacher VJ, Wu CY, Benet LZ. Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. ***Mol Carcinog***. 1995 Jul; 13(3): 129-34.

71. Wagner JG. Biopharmaceutics: absorption aspects. ***J Pharm Sci***. 1961 May; 50: 359-87.

72. Watkins PB, Wrighton SA, Schuetz EG, Molowa DT, Guzelian PS. Identification of glucocorticoid-inducible cytochrome P-450 in the intestinal mucosa of rats and man**.** ***J Clin Invest***. 1987 Oct; 80(4): 1029-36.

73. Westlind A, Malmebo S, Johansson I, Otter C, Andersson TB, Ingelman-Sundberg M *et al*. Cloning and tissue distribution of a novel human cytochrome P450 of CYP3A subfamily, CYP3A4. Biochem ***Biophys Res Commun***. 2001 Mar16; 281(5): 1349-55.

74. Yee S. In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestine) absorption in man—fact or myth. ***Pharm Res***. 1997 Jun;14(6):763-6.

Anexo 1 – Parecer do CEP/FOP para o estudo 1.

UNICAMP

COMITÊ DE ÉTICA EM PESQUISA

UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ODONTOLOGIA DE PIRACICABA

CERTIFICADO

Certificamos que o Projeto de pesquisa intitulado "Estudo de bioeqüivalência entre comprimidos contendo 400 mg de carbamazepina do Laboratório Teuto-Brasileiro® e comprimidos contendo 400 mg de carbamazepina da Novartis®", sob o protocolo nº **019/2002**, da Pesquisadora ***ANA PAULA DEL BORTOLO RUENIS***, sob a responsabilidade do Prof. Dr. **PEDRO LUIZ ROSALEN**, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa – FOP.

Piracicaba, 03 de abril de 2002

We certify that the research project with title "Bioequivalence study of 400 mg carbamazepine tablets by laboratório teuto-brasileiro® and 400 mg carbamazepine tablets by novartis®", protocol nº **019/2002,** by Researcher ***ANA PAULA DEL BORTOLO RUENIS***, responsibility by Prof. Dr. **PEDRO LUIZ ROSALEN**, is in agreement with the Resolution 196/96 from National Committee of Health/Health Department (BR) and was approved by the Ethical Committee in Resarch at the Piracicaba Dentistry School/UNICAMP (State University of Campinas).

Piracicaba, SP, Brazil, April 03 2002

Prof. Dr. Antonio Wilson Sallum
Diretor
FOP/UNICAMP

Prof. Dr. Antonio Bento Alves de Moraes
Coordenador
CEP/FOP/UNICAMP

Anexo 2 - Parecer do CEP/FCM para os estudos 2 e 3.

UNICAMP

FACULDADE DE CIÊNCIAS MÉDICAS
COMITÊ DE ÉTICA EM PESQUISA
✉ Caixa Postal 6111
13083-970 Campinas, SP
☎ (0__19) 3788-8936
fax (0__19) 3788-8925
cep@head.fcm.unicamp.br

CEP, 29/10/01
(Grupo II)

PARECER PROJETO: N° 190/2001

I-IDENTIFICAÇÃO:

PROJETO: **"EFEITOS SOBRE A MICROBIOTA ORAL E NASAL DE INDIVÍDUOS SADIOS SUBMETIDOS A UMA DOSE ÚNICA DE 500MG DE CLARITOMICINA MERCK COMPARADA A UMA DOSE ÚNICA DE 500MG DE CLARITOMICINA ABBOT"**
PESQUISADOR RESPONSÁVEL: Eduardo Abib Júnior
INSTITUIÇÃO: Synchropar/Clínica São Lucas
APRESENTAÇÃO AO CEP: 16/08/2001

II - OBJETIVOS

Correlacionar a farmacocinética das formas comerciais da claritromicina 500 mg, a Claritromicina- do Merck S.A. Indústrias químicas e o Klaricid- Abbot laboratórios do Brasil Ltda., com usa eficácia na redução da microbiota oral e nasal de voluntários saudáveis, em diferentes períodos de tempo.

III - SUMÁRIO

Trata-se estudo aberto, randomizado, cruzado onde voluntários receberão as 2 formulações do medicamento claritromicina (cujas concentrações séricas são equivalentes, segundo o autor) em 2 períodos distintos. Vinte e quatro voluntários sadios, de ambos os sexos, com idade entre 18 e 45 anos, receberão dose única de 500mg e será avaliada a contagem de microorganismos no esfregaço nasal e na saliva antes da administração da droga, e após 80 min, 120min, 360min, e 720 min da administração. Previamente ao estudo serão avaliadas as condições de saúde dos sujeitos e colhidos exames para bioquímica e sorologias. Os sujeitos necessitarão de internação por 2 períodos de 36 h e terão seus custos remunerados com a quantia de R$ 25,00. O estudo prevê também seguro por um período de 6 meses após o início da pesquisa.

IV - COMENTÁRIOS DOS RELATORES

O apresenta-se nos moldes estabelecidos pelas resoluções 196 e 251 do CNS/MS. A justificativa do protocolo centra-se no interesse em estabelecer que a claritromicina pode ser um substituto seguro de outras drogas na profilaxia odontológica da endocardite bacteriana, de forma que parece haver uma relação custo-benefício favorável. O Termo Consentimento Livre e Esclarecido encerra as informações necessárias aos sujeitos.

SITUAÇÃO DO PROJETO: APROVADO

V - PARECER DO CEP

O Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP, após acatar os pareceres dos membros-relatores previamente designados para o presente caso e atendendo todos os dispositivos das Resoluções 196/96 e 251/97, bem como ter aprovado o Termo do Consentimento Livre e Esclarecido, assim como todos os anexos incluídos na Pesquisa, resolve aprovar sem restrições o Protocolo de Pesquisa supracitado.

VI - INFORMAÇÕES COMPLEMENTARES

O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (Res. CNS 196/96 – Item IV.1.f) e deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado (Item IV.2.d).

Pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que aprovou (Res. CNS Item III.1.z), exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade de regime oferecido a um dos grupos de pesquisa (Item V.3.).

O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS Item V.4.). É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projeto do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve envia-las também à mesma junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial (Res. 251/97, Item III.2.e)

Relatório final deve ser apresentado ao CEP, ao término do estudo.

VII - DATA DA REUNIÃO

Aprovado em 29 de outubro de 2001.

A ser homologado na XI Reunião Ordinária do CEP/FCM, em 13 de novembro de 2001.

Prof. Dr. Sebastião Araújo
PRESIDENTE do COMITÊ DE ÉTICA EM PESQUISA
FCM / UNICAMP

Anexo 3 –Comprovante de submissão a revista Qualis A Internacional – Estudo 3

YAHOO! Mail

Imprimir - Fechar janela

De: "Francisco Groppo" <fcgroppo@fop.unicamp.br>
Para: ap_ruenis@yahoo.com.br
Assunto: ENC: [PSJnet] â€™Sâ€œâ€"â€¢Ã'Â□WËtÃ□ËtÃµâ€šÃŒ â€™ÃŠâ€™m
Data: Fri, 6 Feb 2004 08:58:40 -0500

Prof. Dr. Francisco C. Groppo
Ã□rea de Farmacologia, Anestesiologia e TerapÃªutica
Fac. Odont. de Piracicaba - UNICAMP
Av Limeira 901 - CEP 13414 903
Piracicaba - SÃ£o Paulo
Fone 19 3412 5310 - FAX 19 3412 5208

-----Mensagem original-----
De: â€œÃ°â€"{â€"Ã²Å wâ€°Ã¯PSJnet [mailto:eronb@pharm.or.jp]
Enviada em: Thursday, February 05, 2004 9:02 PM
Para: fcgroppo@fop.unicamp.br
Cc: terasaki@mail.pharm.tohoku.ac.jp
Assunto: [PSJnet] æ‹…å½"ç·¨é›†å§"å"¡ã□®é€šçŸ¥

ã□"ã□®ãƒ¡ãƒ¼ãƒ«ã□¯è‡ªå‹•é□ä¿¡ã□§ã□™ã€,è¿"ä¿¡ã□¯ã□—ã□ªã□„ã□§ä¸‹ã□•ã□„ã€,
This mail is auto transmission. Please do not carry out a reply.

æ¬¡ã□®è«-æ-‡ã□®æ‹…å½"æ±°å®šã□—ã□¾ã□—ã□Ÿã€,

æ‹…å½": Tetsuya Terasaki

Name: Francisco Carlos Groppo
Title: A PK/PD approach on the effects of clarithromycin against oral and nasal microbiota of healthy volunteers
è«-æ-‡é¡žåˆ¥: Regular Article
Manuscript ID: b040019
Journal: BPB

Anexo 4 - Comprovante de submissão a revista Qualis A Internacional – Estudo 2

Successful Submission Confirmation Feb 12, 2004 07:21 07:21

Your manuscript has been successfully uploaded to Journal of Pharmaceutical Sciences. You will receive future communications via e-mail.

Your manuscript number is: **04-066-**

Please make note of your manuscript number. You will receive an e-mail from JPS within 24 hours of the end of this process, confirming receipt of your submission.

Print This Page

Return to the Author Center

Log Out

Anexo 5 - Carta de aceite em revista para o estudo 1.

Declaração

Declaramos para os devidos fins que o trabalho intitulado "**Bioequivalência de Formulações Orais de Carbamazepina Disponíveis no Brasil**" de autoria de Ana Paula Del Bortolo Ruenis, Gilson Cesar Nobre Franco, Sinvaldo Baglie, Luciana Aparecida Tiberti, Luiz Madaleno Franco, Francisco Carlos Groppo e Pedro Luiz Rosalen, foi aceito para publicação na Revista Brasileira de Ciências da Saúde, desde que sejam realizados as correções sugeridas.

João Pessoa, 26 de janeiro de 2004

Prof. Henrique Gil da Silva Nunesmaia
Editor

REBRASA / CCS / UFPB – Cidade Universitária - Fone: (83) 216-7274 • E-mail: rebrasa@ccs.ufpb.br

PROF. PEDRO ROSALEN - UNICAMP URGENTE
(19) 3521 8257