CELSO HENRIQUE DE OLIVEIRA

DOSAGEM DE CONCENTRAÇÕES PLASMÁTICAS DE MEDICAMENTOS ATRAVÉS DE CROMATOGRAFIA LÍQUIDA DE ALTA EFICIÊNCIA ACOPLADA A ESPECTROMETRIA DE MASSA (LC-MS-MS) E SUA APLICAÇÃO EM ESTUDOS DE BIOEQUIVALÊNCIA

Este exemplar corresponde à versão final da Tese de Doutorado, apresentada à de Pós-Graduação da Faculdade de Ciências Médicas - UNICAMP, para obtenção do Título de Doutor em Farmacologia do Médico Celso Henrique de Oliveira.

Campinas, 10 de junho de 2002.

Prof. Dr. Gilberto de Nucci
- Orientador -

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Tese de doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para a obtenção do título de Doutor em Farmacologia.

Orientação: Prof. Dr. Gilberto De Nucci

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A todos o meu mais sincero obrigado.

SUMÁRIO

LISTA DE ABREVIATURAS

- **ANVISA** – Agência Nacional de Vigilância Sanitária
- **ASC** – Área sob a curva farmacocinética da substância analisada
- **CEP** – Comitê de Ética em Pesquisa
- **C_{max}** – Pico de concentração plasmática máxima do medicamento
- **CONEP** – Conselho Nacional de Ética em Pesquisa
- **CLAE ou HPLC** – Cromatografia líquida de alta eficiência
- **FDA** – *Food and Drug Administration* – Agência de controle de medicamentos e alimentos dos Estados Unidos da América
- **LC-MS-MS** – Cromatografia líquida de alta eficiência, acoplada a espectrômetro de massa
- **ESI** – *Electrospray ionization*
- **HU** – Hospital Universitário da Universidade de São Paulo
- **ICB** – Instituto de Ciências Biomédicas da Universidade de São Paulo
- **IS** – Droga utilizada como padrão interno ou *internal standard*
- **Min** – Minuto(s)
- **MRM** – *Multiple Reaction Monitoring*
- ***m/z*** – massa/ carga do íon
- **QC** – Controle de qualidade
- **Tmax** – tempo do pico de concentração máxima da droga
- **UFC** – Universidade Federal do Ceará
- **UNICAMP** – Universidade Estadual de Campinas
- **USP** – Universidade de São Paulo
- **UV** – Ultravioleta

A quantificação de concentrações de fármacos ativos em matrizes biológicas pode ser realizada através de diversas metodologias, sendo a cromatografia líquida de alta eficiência com leitura através de fluorescência ou ultravioleta, a mais frequentemente utilizada na atualidade. No entanto, outras metodologias como a utilização da cromatografia líquida acoplada a espectrômetro de massa (LC-MS-MS) tem aumentado progressivamente, inclusive no Brasil. Fatores implicados nesse aumento são a alta sensibilidade, precisão, exatidão e rapidez do método.

Nesse trabalho, avaliou-se a quantificação de 6 diferentes medicamentos, num total de 7 estudos de bioequivalência, quantificados do plasma humano através de LC-MS-MS na Unidade Analítica Cartesius (São Paulo/SP). Os estudos apresentados demonstraram elevada rapidez, robustez e praticidade com alta precisão e exatidão.

A dosagem de medicamentos em plasma e soro humanos é rotina em grandes centros de pesquisa no mundo, sendo a procura de métodos mais apurados, sensíveis e específicos uma constante.

Diferentes métodos são aplicados na quantificação de drogas e/ou medicamentos em matrizes biológicas humanas. Métodos de quantificação direta e indireta podem ser utilizados, havendo no entanto diferença na sensibilidade e especificidade nos níveis de concentração mínimos de medicamento para sua determinação em cada matriz biológica. Novas metodologias empregadas para esse fim têm conseguido aumentar a sensibilidade e a rapidez da quantificação, acarretando uma diminuição progressiva da concentração mínima do fármaco para a sua determinação. Atualmente existem metodologias que permitem uma sensibilidade da ordem de picogramas ou menos de fármaco por mililitro de amostra.

A realização de dosagens plasmáticas de medicamentos através de cromatografia líquida de alta eficiência (CLAE ou HPLC) acoplada a espectrômetro de massa (LC-MS-MS) trouxe maior sensibilidade e rapidez às mensurações. Esse método no entanto ainda é pouco utilizado pois são considerados dispendiosos economicamente e de manipulação restrita a técnicos altamente qualificados.

Segundo Lagerwerf e cols. (2000), o tempo médio de corrida de uma amostra através de HPLC varia em torno de 10 min (Fluorescência) a 15 min (Ultravioleta - UV) e em torno de 20 min quando da utilização de detecção eletroquímica. Esse tempo no entanto decresce para 3 minutos quando se utiliza a LC-MS-MS. Esses dados demonstram a rapidez da LC-MS-MS se comparada

Apresentar a metodologia de LC-MS-MS empregada para quantificação de fármacos em amostras plasmáticas provenientes de estudos de bioequivalência clínica de medicamentos alopáticos realizados pela Unidade Analítica Cartesius – do Instituto de Ciências Biomédicas (ICB) da Universidade de São Paulo (USP).

1.1. Medicamentos Utilizados:

O presente trabalho se baseou em estudos de bioequivalência realizado em voluntários sadios de ambos os sexos e em hospitais brasileiros. Os pormenores da metodologia utilizada e detalhes da parte clínica realizada são apresentados nos artigos publicados **[Anexos 1 a 6]**. Todos os procedimentos foram submetidos a apreciação de Comitês Independentes de Ética em Pesquisa Clínica. Os estudos apresentados são os dos seguintes medicamentos:

- **Amoxicilina** – suspensão oral de 250 mg/5mL dos laboratórios Medley S/A Indústria Farmacêutica (formulação teste) e SmithKline Beecham Laboratórios Ltda. (Amoxil®, formulação referência) **[Anexo 1]**
- **Amoxicilina** - cápsulas 500 mg dos laboratórios Medley S/A Indústria Farmacêutica (formulação teste) e SmithKline Beecham Laboratórios Ltda. (Amoxil®, formulação referência) **[Anexo 1]**
- **Cefadroxil** – cápsulas de 500 mg dos laboratórios Eurofarma Laboratórios Ltda. (Cefadroxila®, formulação teste) e Bristol-Myers Squibb (Cefamox®, formulação referência) **[Anexo 2]**
- **Terbinafina** – comprimidos de 250 mg dos laboratórios Medley S/A Indústria Farmacêutica (formulação teste) e Novartis Biociências S/A (Lamisil®, formulação referência) **[Anexo 3]**

1.3. Tipos de extração:

Os métodos para extração de fármacos de matrizes biológicas utilizados nos estudos foram a extração *líquido-líquido*, em *fase sólida* e por *precipitação de proteínas*.

1.3.1. Extração líquido-líquido:

A extração líquido-líquido dos produtos analisados foi feita para os estudos da terbinafina, amlodipina e nevirapina **[Anexos 3, 5 e 6]**.

Basicamente, essa metodologia de extração envolve diferentes passos. Um modelo padrão de extração líquido/líquido é descrito abaixo:

Coloca-se cada amostra e o respectivo padrão interno em tubos de vidro específicos e após homogeinização, adiciona-se uma mistura de solventes orgânicos como por exemplo a mistura de éter etílico e hexano na proporção de 80:20 (v/v). Dependendo da droga, o pH do plasma pode – ou não, ser ajustado a valores de pH ácido ou básico. Após nova homogeinização, remove-se a camada orgânica e transfere-se esta solução para novos tubos onde o solvente será evaporado com a ajuda de um fluxo de Nitrogênio a uma temperatura de 37°C. O resíduo ou extrato é ressuspenso num volume conhecido de uma mistura de solventes (por exemplo a própria Fase Móvel da corrida cromatográfica sendo posteriormente transferidos para os *microvials* e posteriormente ao injetor automático do aparelho onde a substância será finalmente injetada no cromatógrafo líquido, separada através da coluna cromatográfica e detectada por espectrometria de massas.

1.3.3. Extração por precipitação de proteínas:

A extração por precipitação de proteínas foi utilizada para a determinação dos níveis plasmáticos da metildopa **[Anexo 4]**. Este tipo de extração é a forma mais simples de extração pois possui somente 1 passo principal.

Basicamente, na quantificação da metildopa cada amostra de plasma foi colocada junto a água, solução de metanol/água/padrão interno e ácido perclórico. Após homogeinização, as impurezas lipofílicas eram então extraídas utilizando diclorometano. A mistura era então misturada e centrifugada, sendo a parte superior transferida para os *microvials*. A amostra então é colocada no amostrador automático, injetada no cromatógrafo e detectada no espectrômetro de massas.

1.4. Cromatografia líquida de alta eficiência:

Todos os estudos foram realizados utilizaram uma coluna analítica de sílica de fase reversa (fase móvel polar) do tipo C_{18} de marca Jones Chormtography modelo Genesis com partículas de 4 μm, diâmetro interno da coluna de 4.6 mm e um comprimento de 150 mm para a separação das amostras biológicas previamente extraídas. No caso da separação da metildopa, a coluna acima foi precedida de uma pré-coluna de diâmetro interno de 2.1 mm e 10 mm de comprimento.

A cromatografia apresentou tempos totais de corridas que variavam de 3 a 5.5 minutos. O fluxo típico oscilava entre 0,5 e 1,0 ml/min.

1.6. Escolha do padrão interno (IS):

Para a escolha do padrão interno adequado, a fim de compensar eventuais perdas durante o processo de extração do analito, deve-se levar em conta a similaridade na estrutura química entre o analito e o padrão interno (análogos), para isso deve-se escolher um padrão interno contendo os mesmos grupo funcionais do analito; esse procedimento pode evitar problemas durante a fase de extração da matriz biológica. No entanto, as condições de ionização devem ser diferentes.

Compostos marcados isotopicamente, por exemplo Isótopos deuterados (o próprio analito contendo átomos de deutério na estrutura) podem ser utilizados como padrão interno, como no estudo da metildopa **[Anexo 4]**. No entanto, este tipo de compostos nem sempre são acessíveis comercialmente e quando existem o custo dos mesmos torna inviável a utilização em análises de rotina.

1.8. Validação do método e controle de qualidade (QC):

De acordo com as Normas do *Food and Drug Administration* - FDA (1998b), os seguintes pontos são necessários para a validação do método analítico e controle de qualidade das corridas das amostras:

- Limite Mínimo de Quantificação (LOQ) com variação na sua precisão e exatidão de até ± 20%
- Controle de Qualidade (QC) com variação na sua precisão e exatidão de até ± 15%
- Curva de calibração com pelo menos 6 pontos
- Coeficiente de correlação ≥ 0.95
- Corrida de um novo QC a cada 10 amostras desconhecidas
- Critério de rejeição da corrida analítica:
  1. 30% do total de QCs ou
  2. 100% de um mesmo QC (mesma concentração; ex.: todos os QCAs excluídos).

Além disso, para a escolha das concentrações dos controles de qualidade, devem ser observados os seguintes critérios:

- QCA ⟨ ou = 3x LOQ
- QCB entre QCA e QCC
- QCC entre 75 e 90% do ponto mais alto da curva de calibração
- Curva de calibração deve cobrir a faixa de concentrações esperada para a substância analisada.

Os critérios de aceitação dos testes de estabilidade são um número mínimo de 3 repetições com variação máxima de ± 20% na precisão e exatidão observados. A estabilidade do padrão interno não é avaliada (com exceção do teste em amostrador automático).

1.10. Limite de quantificação (LOQ):

A determinação do limite de quantificação (LOQ) era padronizada para que se garantisse um mínimo de 1% do pico de concentração plasmática ($C_{máx}$), havendo no entanto que se considerar das dificuldades de cada método empregado, tanto a nível de extração, corrida no HPLC e detecção no espectrômetro de massa.

1.11. Ética em pesquisa Clínica:

Os estudos realizados nesse trabalho foram submetidos a aprovação de Conselhos de Ética em Pesquisa (CEP), seja na Universidade Estadual de Campinas (UNICAMP), Universidade Estadual de São Paulo (USP) - no Instituto de Ciências Biomédicas e/ou Hospital Universitário (HU) ou mesmo na Universidade Federal do Ceará (UFC). Todos os voluntários assinaram o consentimento pós-informação para a realização dos procedimentos de estudo de bioequivalência e coleta de amostras sangüíneas.

A constante de eliminação (ke) de cada medicamento analisado foi determinada a partir de regressão linear de um mínimo de 3 pontos da fase de eliminação da droga em padrão log-linear. A meia-vida plasmática ($t_{1/2}$) foi derivada dessa constante ke a partir de fórmula baseada em logarítmo natural de 2, como apresentado abaixo:

$$t_{1/2} = \ln(2) / ke$$

A concentração plasmática máxima ($C_{máx}$) observada e o tempo necessário para a sua ocorrência ($T_{máx}$) foram determinados a partir de observação direta na curva de farmacocinética (concentração *vs* tempo) de cada medicamento.

Para o cálculo da Área Sob a Curva (ASC) farmacocinética de cada medicamento, foi realizada pela transformação logarítmica dos valores obtidos entre os tempos zero e o último tempo quantificado ou infinito ($AUC_{0\text{-}last}$ e $AUC_{0\text{-}inf}$) e o $C_{máx}$. Para o cálculo das áreas, foi utilizado a método trapezoidal de determinação de áreas. A extrapolação até o infinito foi realizada pela adição do valor encontrado quando do último tempo de quantificação observado (C_{last}) dividido pelo ke, à área de AUC_{last} conforme a fórmula abaixo:

$$AUC_{0\text{-}\infty} = AUC_{last} + C_{last} / ke$$

A quantificação das amostras de medicamentos foi realizada de acordo com os parâmetros apresentados na **Tabela 1** e nos **Anexos 1 a 6**. Nesses anexos encontram-se os dados e resultados, gráficos, tabelas e análise estatística correspondentes.

TABELA 1	Amoxicilina	Cefadroxil	Terbinafina	Metildopa	Amlodipina	Nevirapina
Nome do Padrão Interno (IS) escolhido	Cefadroxil	Amoxicilina	Naftifina	Dopa-fenil-D3	Desipramina	Dibenzepina
Matriz biológica	Plasma	Plasma	Plasma	Plasma	Plasma	Plasma
Tipo de Extração	Sólida/Líquida	Sólida/Líquida	Líquida/Líquida	Precipitação pts	Líquida/Líquida	Líquida/Líquida
Modelo Micromass	Quattro LC	Quattro LC	Quattro II	Quattro Ultima	Quattro LC	Quattro II
Tipo de Electrospray	+	+	+	+	+	+
Transição Analito (*m/z*)	366,0 > 349,2	364,0 > 207,8	292,2 > 140,7	212,1 > 166,1	409,0 > 238,1	267 > 225
Transição Padrão Interno (*m/z*)	364,0 > 207,8	366,0 > 349,2	288,2 >116,9	201,2 > 154,2	267,1 > 236,2	296 > 251
Volt. Cone analito/IS (V)	20,2/20,2	20,2/20,2	35/25	25/15	20/20	25/25
Energia Colisão analito/IS (eV)	10/10	10/10	15/12	15/16	11/15	23/15
Dwell time (s)	0,5/0,5	0,5/0,5	0,5/0,5	0,8/0,8	0,8/0,8	0,5/0,5
Tempo de corrida (min)	5,0	5,0	5,0	5,5	5,0	5,0
Tempo de retenção (analito/IS; min)	2,63/2,63	2,56/2,56	2,96/2,82	3,4/3,4	3,4/3,6	2,7/3,3
Limite quantificação (LOQ; ng/mL)	50,0	50,0	5,0	20,0	0,1	10,0
Precisão % (LOQ)	115,4	110,4	109,1	103,0	109,3	106,3
Exatidão % (LOQ)	93,5	94,3	99,4	110,7	100,0	106,0

Tabela 1 - Características gerais da metodologia empregada através de cromatografia líquida de alta eficiência acoplada a espectrômetro de massa (LC-MS-MS) para quantificação dos diferentes medicamentos.

A quantificação das amostras de um fármaco inicia-se pela avaliação do melhor método de extração da matriz biológica, seja ela plasma, soro, sangue total, etc. No presente estudo, todas as amostras apresentadas foram extraídas de plasma humano.

De acordo com Lagerwerf e cols. (2000), a extração das amostras ocorre pela necessidade de se evitar o fenômeno chamado de "supressão iônica" que pode ocorrer no espectrômetro de massa, induzido por componentes que se encontram na matriz biológica tais como açúcares, lipídeos, aminoácidos, proteínas, íons metálicos, etc. Esses componentes podem ser co-eluídos na cromatografia com o analito, e, mesmo havendo um limite de influência – acarretado pela competição na ionização ou pelo limite da gota na produção de íons, podem interferir na sua ionização.

Apesar desses componentes permanecerem indetectáveis pelo espectrômetro devido à sua especificidade pelo analito - determinada pela detecção por monitoração de reações múltiplas (MRM), eles realmente podem afetar a exatidão, precisão e a reprodutibilidade do método.

A supressão iônica é conhecida também como efeito matriz. O efeito matriz é um fenômeno muito comum nas análises de LC-MS/MS pois leva em consideração o ambiente químico no qual encontra-se o analito. O efeito matriz pode ser negativo, caso exista uma supressão do sinal, ou positivo, caso exista um incremento do sinal. O experimento para verificação deste efeito consiste na contaminação de uma amostra de plasma branco previamente extraído e ressuspendido num volume fixo do solvente apropriado (extrato). O melhor

Um exemplo comum de fase móvel utilizada para estudos com LC-MS-MS é aquela que contém água com algum outro solvente orgânico como metanol ou acetonitrila como modificadores orgânicos. A adição de ácidos ou bases na fase móvel das análises por espectrometria de massas se faz necessário pelo fato destes compostos auxiliarem na ionização das substâncias a serem analisadas por esta razão são chamados de aditivos (ex. solução de hidróxido de amônia ou ácido acético ou fórmico). O uso de sais orgânicos como acetato de amônio são bastante utilizados a fim induzir a formação de adutos visando um aumento de sensibilidade na detecção por espectrometria de massas. São chamados de adutos os íons que apresentam a estrutura [M-X] onde X é um íon metálico por exemplo sódio, potássio, amônio, etc. O uso de sais orgânicos junto com os seus respectivos ácidos também são bastante utilizados para fixar o pH da fase móvel (efeito tampão). Todas as amostras analisadas nos estudos de bioequivalência apresentados nesse trabalho foram separadas utilizando-se eluição isocrática (composição constante da fase móvel).

Diversos métodos espectrométricos podem ser utilizados na quantificação de fármacos. Além da espectrometria de massa, a espectrometria no infravermelho, de ressonância magnética de hidrogênio ou de ^{13}C, além da espectroscopia na região do ultravioleta são exemplos de diferentes métodos espectrométricos potencialmente úteis (Silverstein, Bassler & Morrill, 1994).

No entanto, a espectrometria de massas apresenta-se como o método mais versátil e nos últimos anos com o barateamento dos equipamentos, tornou-se uma técnica rotineira nas análises de bioequivalência.

cruzado de fluxo (*electrospray source using a crossflow counter electrode*), podendo ser utilizado com em modo positivo (ESI+) ou negativo (ESI-). Para tal, utiliza-se a protonação ou desprotonação das moléculas analisadas - ou seja, através da 'adição' ou 'subtração' de um próton à molécula em investigação.

Os espectrômetros de massa e os respectivos estudos onde foram utilizados são apresentados a seguir:

- **Micromass model Quattro II** - Utilizado nos estudos de bioequivalência da terbinafina enevirapina **[Anexos 3 e 6]**.
- **Micromass model Quattro LC** - Utilizado nos estudos de bioequivalência da amoxicilina (duas formulações), cefadroxil e amlodipina **[Anexos 1, 2 e 5]**.
- **Micromass model Quattro Ultima** - Utilizado nos estudos de bioequivalência da metildopa **[Anexo 4]**.

De acordo com as especificações do fabricante (Micromass® Ltd.) os aparelhos se diferenciam em alguns pontos específicos, sobretudo pelo sistema de dessolvatação com nitrogênio. O sistema de dessolvatação pode ser através de uma fonte de íons com feixe gasoso em eixo único no modelo Quattro II e em forma de "Z" (z-spray™) no Quattro LC e Quattro Ultima. Representações esquemáticas dos eixos são apresentadas a seguir **[Figuras 1 a 5]**:

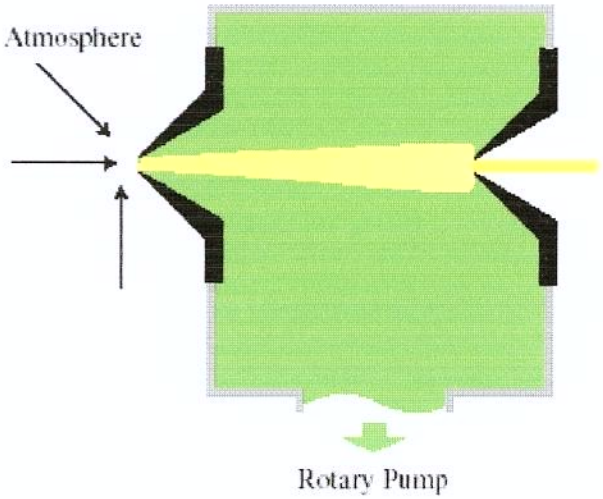


Figura 1: Sistema do cone com um eixo único. Fonte: www.micromass.co.uk

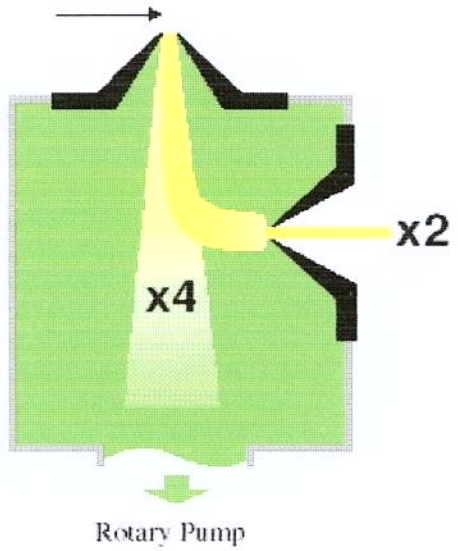


Figura 2: Sistema do cone na forma ortogonal (Z-spray). Fonte: www.micromass.co.uk

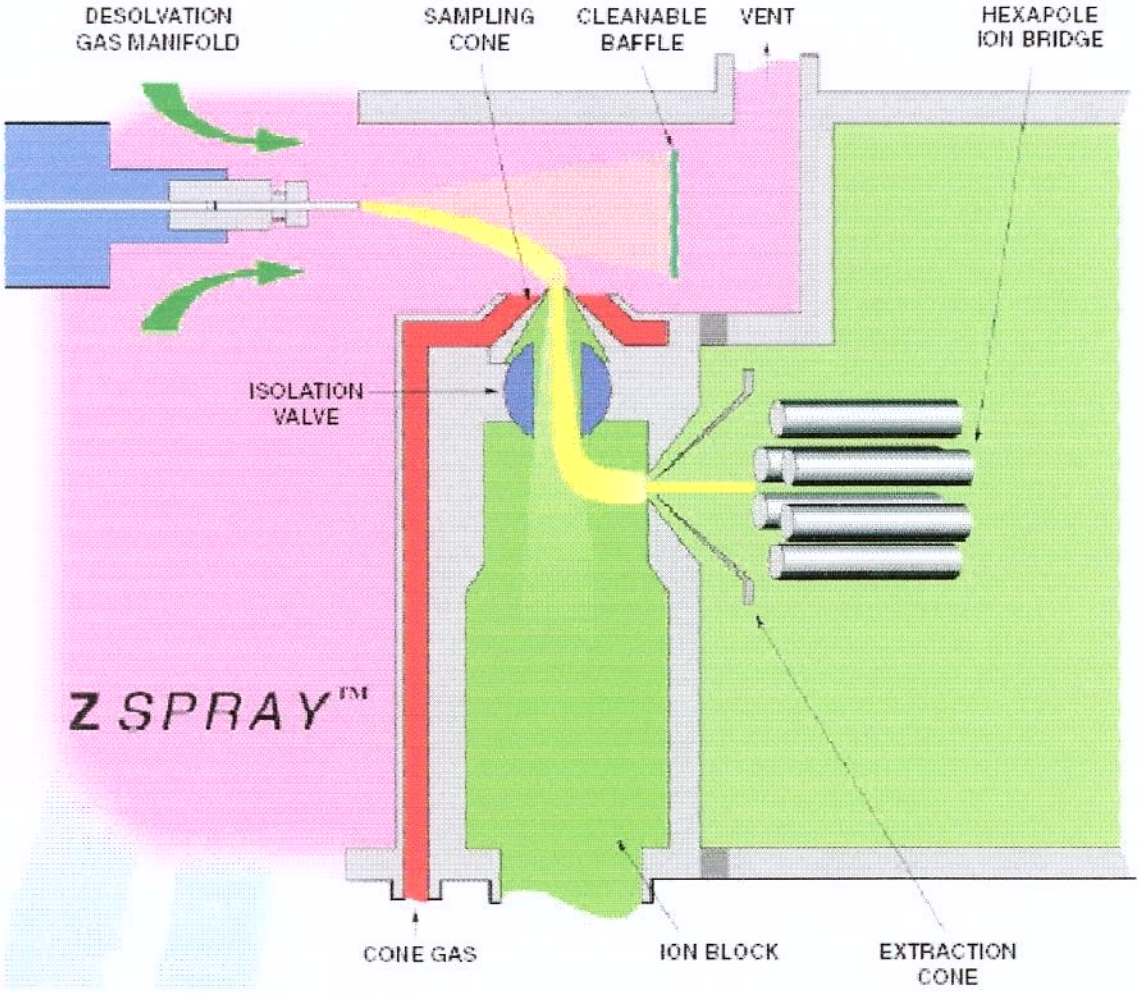
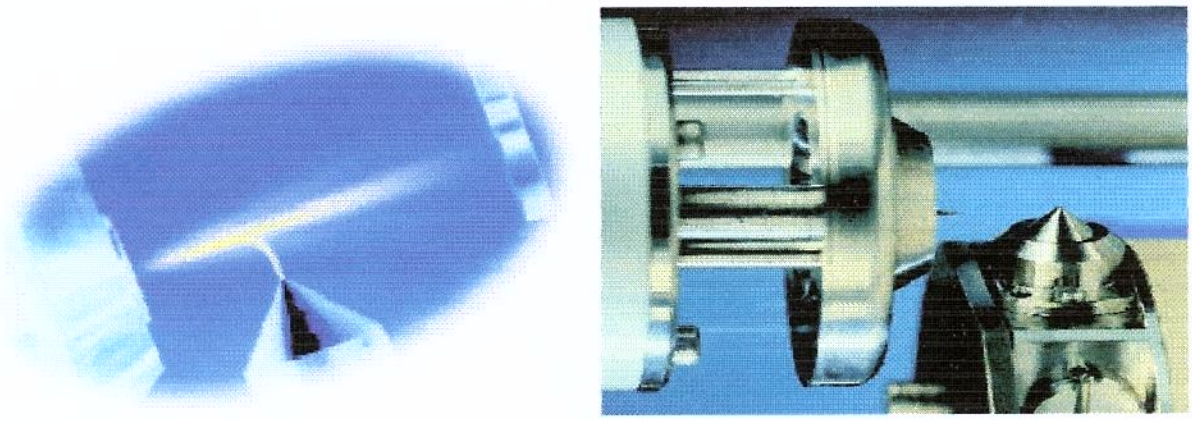


Figura 3: Visão esquemática do eixo ortogonal (Z-spray™). Fonte: www.micromass.co.uk



Figuras 4 e 5: Visão direta do redirecionamento do spray de íons determinado pelo vácuo do cone e a formação em z-spray™. Fonte: www.micromass.co.uk

Assim, após a separação pela cromatografia líquida de alta eficiência, a amostra a ser analisada é introduzida no espectrômetro de massas. A passagem da fase líquida para a gasosa (dessolvatação) é realizada com auxílio do gás nitrogênio, formando-se um "spray". O capilar por onde passa a amostra encontra-se carregado eletricamente daí a origem do nome *electrospray*. O composto assim vaporizado é captado pelo cone do espectrômetro e é introduzido na câmara de vácuo. Os eixos do feixe gasoso podem ser único (reto) no caso do Quattro II ou na forma ortogonal (*Z*-spray™) no caso dos Quatto LC e Ultima. A utilização da técnica de Z-spray™ tem determinado uma maior sensibilidade e simplicidade na quantificação da amostra. A maior sensibilidade deste sistema de introdução de amostras (ortogonal) deve-se, principalmente, à diminuição do ruído proveniente da entrada de partículas não ionizadas na câmara de vácuo. Além disso, os contaminantes podem ser facilmente removidos através da limpeza do anteparo posterior ao feixe gasoso.

Após a passagem pelo cone, o analito penetra em uma série de analisadores de massa dispostos em fileira única (*tandem*) para a quantificação e seleção dos íons. O primeiro compartimento é onde acontece a seleção dos íons precursores – compartimento Q1 (pressão de 10^{-5} mBar). Um segundo compartimento (Q2) apresenta pressão de 10^{-3} mBar e é onde a fragmentação ocorre pela colisão dos íons precursores com moléculas de Argônio (gás de colisão). A partir daí, os íons produto penetram em outro compartimento onde são novamente selecionados e finalmente o íon que sai deste compartimento final é detectado por uma célula fotomultiplicadora. O número de contagens por segundo é registrado na forma de um gráfico (cromatograma) e a área sob esta curva é diretamente proporcional à concentração do analito na amostra, é assim que acontece o processo de quantificação.

Quando se avaliam as características entre a quantificação de fármacos através dos diversos tipos de cromatografia e aquela acoplada ao espectrômetro de massa, observa-se as seguintes diferenças (Lagerwerf et al., 2000):

Cromatografia:

- Detetores: Cromóforo (UV), fluoróforo (fluorescência) ou por eletroquímica
- Seletividade: variável (+ a +++)
- LOQ típico: de 100 pg/mL (eletroquímica) a 10.000 pg/mL (UV)
- Tempo de corrida: de 10 min (Fluorescência) a 20 min (eletroquímica)

LC-MS-MS:

- Detetor: por ionização (MS-MS)
- Seletividade: elevada (++++)
- LOQ típico: 50 pg/mL
- Tempo de corrida: 3 a 5 min

Os resultados obtidos nos estudos apresentados demonstraram uma alta sensibilidade e rapidez do método, com quantificação de concentrações plasmáticas baixas e com tempos de corrida analítica que variaram de 3,5 a 5,5 minutos. Apesar de não terem sido avaliados os custos da utilização de tal metodologia, observa-se que a LC-MS-MS demonstra grande robustez e praticidade para quantificações em larga escala como estudos de bioequivalência, justificando a sua utilização.

Os estudos apresentados demonstram uma grande aplicabilidade do uso de cromatografia líquida de alta eficiência acoplada a espectrômetro de massa para a determinação de medicamentos em plasma humano. Essa aplicabilidade pode ser utilizada com vantagens em estudos de bioequivalência farmacêutica devido ao baixo tempo para a quantificação das amostras e à precisão e exatidão do método.

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Comparative bioavailability of 4 amoxicillin formulations in healthy human volunteers after a single dose administration

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Key words
amoxicillin – bioavailability – mass spectrometer – chromatography

Abstract. Objective: To compare the bioavailability of two amoxicillin oral suspension (250 mg/5 ml) formulations and two amoxicillin capsule (500 mg) formulations (Amoxicilina from Medley S/A Indústria Farmaceûtica, Brazil, as test formulations and Amoxil from SmithKline Beecham Laboratórios Ltda., Brazil, as reference formulations) in 48 volunteers of both sexes. Material and methods: The study was conducted open with a randomized two-period crossover design and a one-week washout period. Plasma samples were obtained over a 12-hour interval. Amoxicillin concentrations were analyzed by combined reversed phase liquid chromatography and tandem mass spectrometry (LC-MS-MS) with positive ion electrospray ionization using the selected ion monitoring method. From the amoxicillin plasma concentration vs. time curves the following pharmacokinetic parameters were obtained: AUC_{last}, $AUC_{0-\infty}$ and C_{max}. Results: Geometric mean of Amoxicilina/Amoxil 250 mg/5 ml individual percent ratio was 103.70% for AUC_{last}, 103.15% for $AUC_{0-\infty}$ and 106.79% for C_{max}. The 90% confidence intervals were 97.82 – 109.94%, 97.40 to 109.24%, and 96.38 – 118.33%, respectively. Geometric mean of Amoxicilina/Amoxil 500 mg capsule individual percent ratio was 93.26% for AUC_{last}, 93.27% for $AUC_{0-\infty}$ and 90.74% for C_{max}. The 90% confidence intervals were 85.0 – 102.33%, 85.12 – 102.31%, and 80.14 – 102.73%, respectively. Conclusion: Since the 90% CI for both C_{max}, AUC_{last} and $AUC_{0-\infty}$ were within the 80 – 125% interval proposed by the Food and Drug Administration, it was concluded that Amoxicilina 250 mg/5 ml oral suspension and Amoxicilina 500 mg capsule were bioequivalent to Amoxil 250 mg/5 ml oral suspension and to Amoxil capsule 500 mg, respectively, with regard to both the rate and extent of absorption.

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Introduction

Amoxicillin [[2S-[2α,5α,6β(S*)]]-6-[[Amino(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid] is an oral, semisynthetic antibiotic related to penicillin. The penicillin nucleus consists of a thiazolidine ring connected to a β-lactam ring to which is attached a side chain. The side chain determines the pharmacological and antibacterial properties of amoxicillin. Amoxicillin is commercially available in capsules and tablets containing 250 and 500 mg (as amoxicillin free base) for oral administration. It is also commercially available for oral administration as suspensions containing 125 and 250 mg/5 ml.

The objective of this study was to compare in healthy volunteers, the pharmacokinetic profiles and to evaluate the bioequivalence of two test formulations of 250 (oral suspension of 250 mg/5 ml) and 500 mg (capsules) of amoxicillin, produced by Medley S/A Indústria Farmacêutica, Brazil (test formulation). The test formulations were compared to two commercial formulations of 250 mg (oral suspension of 250 mg/5 ml) and 500 mg (capsules) of amoxicillin (Amoxil) manufactured by SmithKline Beecham Laboratórios Ltda., Brazil (reference formulation).

Subjects, material and methods

Clinical protocol

Forty-eight healthy volunteers of both sexes (24 male and 24 female) aged between 18 and 50 years and with body weights within

15% of the ideal body weight were selected for the study. They were divided into 2 different groups (A and B). Group A volunteers were divided into 2 subgroups, composed of male (n = 12; 32.3 ± 6.4, mean ± SDM; range 24 – 43 years), height between 163 and 187 cm (173.5 ± 6.8 cm), weighing between 60.5 and 95.5 kg (75.7 ± 9.6 kg) and female volunteers (n = 12; 28.7 ± 5.8 years; range: 21 – 39 years), height between 147 and 168 cm (159.2 ± 5.6 cm), weighing between 52.0 and 74.5 kg (61.3 ± 6.2 kg). Each subject in Group A received both amoxicillin oral suspension formulations.

Group B volunteers were also divided into 2 subgroups, composed of male (n = 12; 33.2 ± 7.16; range: 20 – 41 years), height between 164 and 190 cm (172.3 ± 7.2 cm), weighing between 58 and 83 kg (70.2 ± 8.7 kg) and female volunteers (n = 12; 31.0 ± 5.0 years; range: 22 – 39 years), height between 150.5 and 170 cm (160.2 ± 5.7 cm), weighing between 47 and 98.5 kg (63.2 ± 14.5 kg). Each subject in Group B received both amoxicillin capsule formulations.

All subjects gave written informed consent and the Campinas State University Ethics Committee approved the clinical protocol. All volunteers were healthy as assessed by physical examination, ECG and the following laboratory tests: blood glucose, urea, creatinine, AST, ALT, alkaline phosphatase, γ-GT, total bilirubin, albumin and total protein, trygliceride, total cholesterol, hemoglobin, hematocrit, total and differential white cell counts and routine urinalysis. All subjects were negative for HIV, HBV (except for serological scar) and HCV.

The study was conducted in an open randomized two-period crossover balanced design with a one-week washout period between the doses. During each period, the volunteers were hospitalized at 11:00 p.m. having already had a normal evening meal, and, after an overnight fast, they received a single 250 or 500 mg amoxicillin dose of either formulation at 6:00 a.m. The oral suspension (250 mg/5 ml) was given by instillation with a syringe directly into the volunteer's mouth. Water (200 ml) was given immediately after drug administration. All volunteers were then fasted for 4 h following the drug administration, after which a standard lunch was consumed and an evening meal was provided 10 h after dosing. No other food was permitted during the "in-house" period. Liquid consumption was permitted ad libitum after lunch but xanthine-containing drinks including tea, coffee, and cola were avoided.

Systolic and diastolic arterial pressure (measured non-invasively with a sphygmomanometer), heart rate and temperature were recorded just before and hourly after drug administration.

Formulations

The following test formulations were employed: Amoxicilina 250 mg/5 ml (lot number AXC02/00-1, expiration date 02/2002) and Amoxicilina capsules 500 mg (lot number 9909342, expiration date 10/2001). The details of the reference formulations are as follows: Amoxil 250 mg/5 ml oral suspension (lot number BE0015, expiration date 05/2002) and Amoxil 500 mg capsules (lot number BC0037, expiration date 02/2002).

Drug analysis

Blood samples (8 ml) from a suitable antecubital vein were collected into EDTA containing tubes before and 0.25, 0.5, 1, 1.5, 2, 3, 4, 6 and 8 h after the administration of each amoxicillin suspension formulation (250 mg/5 ml). When the capsule formulation (500 mg) was administered, two further samples were collected at 10 and 12 h. The blood samples were centrifuged at 3500 rpm for 10 min at room temperature and the plasma decanted and stored at –20°C until assayed for their amoxicillin content. All samples from a single volunteer were analyzed on the same day in order to avoid interassay variation. Amoxicillin and the internal standard cefadroxil were extracted from volunteer's plasma by solid-phase extraction using Oasis cartridges (Waters Corp., USA). The compounds were eluted with a small volume of mobile phase, an aliquot of which was analyzed by combined reversed phase liquid chromatography tandem mass spectrometry with positive ion electrospray ionization using selected daughter ion monitoring (MRM).

Briefly, 0.5 ml of purified water was added to a glass tube, followed by plasma sample (50 µl) and internal standard (50 µl of

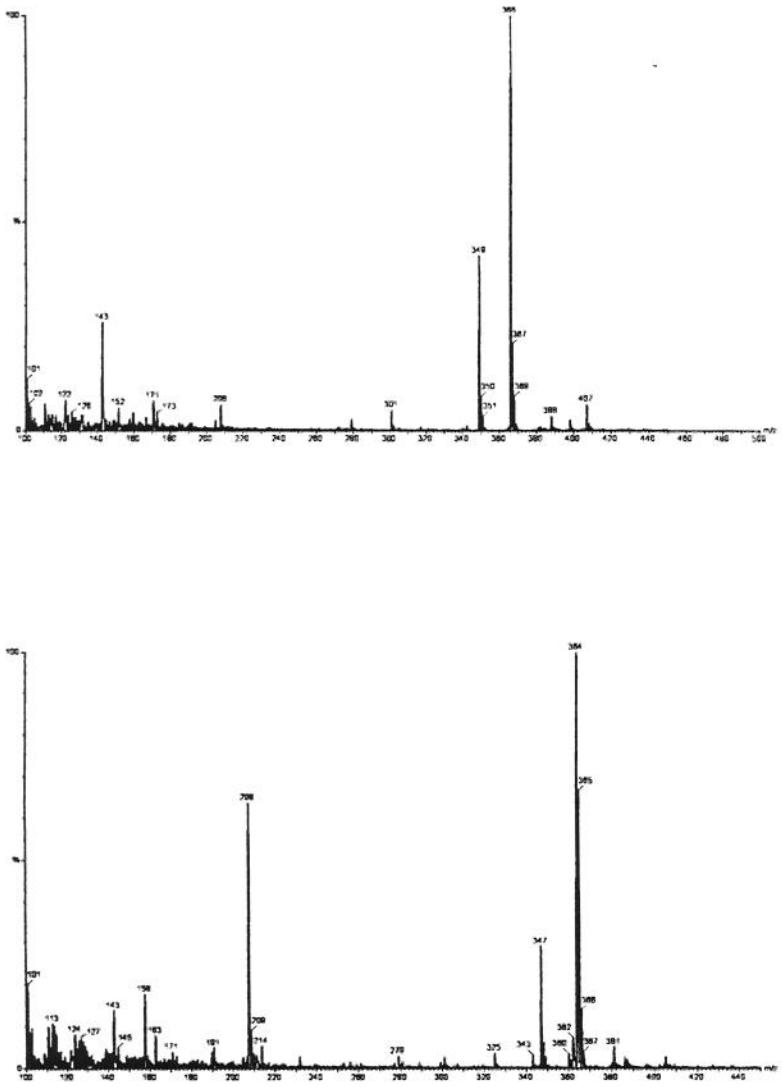

Figure 1. Selected ion spectrograms with respective molecular protonated ions; a: amoxicillin, b: cefadroxil.

cefadroxil 4 µg/ ml). The tube was vortex-mixed for 10 sec and allowed to stand at room temperature for 5 min. Oasis SPE cartridges were preconditioned with methanol (1 ml) and purified water (1 ml) and this was followed by the sample application. After two 2 ml water washings, the column was dried by vacuum suction and the cartridges washed with acetonitrile 50% + 10 mM formic acid (2 ml) and an aliquot (200 µl) was transferred to microvials.

Chromatographic conditions

Chromatography was performed on a Genesis C18 4 µm analytical column (150 mm × 4.6 mm i.d.) fitted with a guard column of the same material. Chromatography was performed isocratically using a mobile phase A (50% acetonitrile containing 10 mM formic acid) at a flow rate of 0.5 ml/min. The pressure of the system was about 70 – 90 bar with a new guard column in place. The column was operated at a temperature of 26° C and a column switch was employed to divert column eluant to waste at appropriate times. A split of the column eluant of approximately 1 : 10 was included so that only approximately 50 µl/min entered the mass spectrometer. The temperature of the autosampler was maintained at 5° C and the injection volume was 20 µl.

Mass-spectrometric conditions

The drug analysis data were acquired and elaborated with MassLynx (v 3.2) running under Windows NT (v 4.0) on a Pentium PC. The total run time was set for 4.0 min and the typical standard retention times were 2.57 min for both amoxicillin and cefadroxil. The mass spectrometer (Micromass model Quattro LC) equipped with electrospray source using a crossflow counter electrode) was run in positive mode (ES+) and set with a multiple reaction mode (MRM) of the following ions 366.0 > 349.2 and 364.0 > 207.8 for amoxicillin and cefadroxil, respectively. For both amoxicillin and cefadroxil the dwelling time, the cone voltage, the collision energy and gas pressure (Argon) were 0.5 sec, 20.2 Volt, 10 eV and 3×10^{-3} bar, respectively.

Pharmacokinetics and statistical analysis

The first-order terminal elimination rate constant (ke) was estimated by linear regression from the points describing the elimination phase in a log-linear plot. Half-life ($t_{1/2}$) was derived from this rate constant ($t_{1/2} = \ln(2)/ke$). The maximum observed plasma concentration (C_{max}) and the time taken to achieve this concentration (t_{max}) were obtained directly from the curves. The areas under the amoxicillin plasma concentration vs. time curves from 0 – 8 h and 0 – 12 h (250

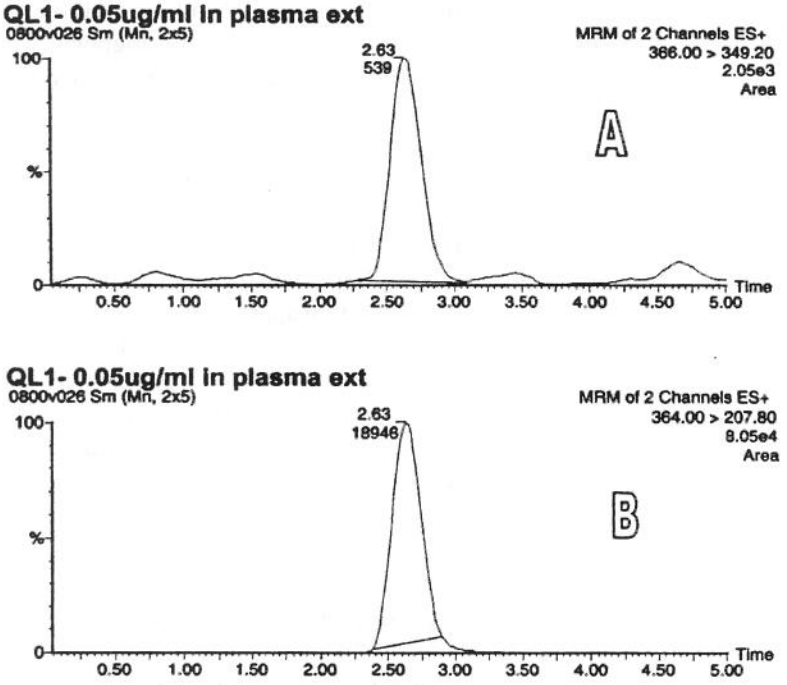

Figure 2. Selected ion chromatograms (MRM). A: MRM of 2 channels ES+: 360.00 > 349.20 for amoxicillin. B: MRM of 2 channels ES+: 364.00 > 207.80 for cefadroxil monohydrate. The retention time and integrated areas are also displayed.

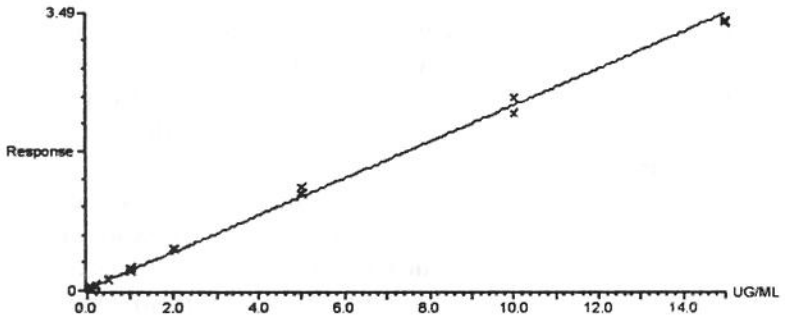

Figure 3. Calibration curve of amoxicillin.

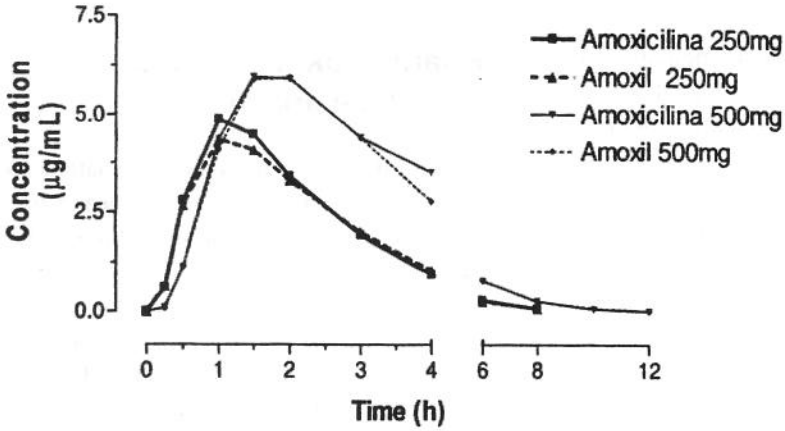

Figure 4. Amoxicillin plasma mean concentrations vs. time profile obtained after the single oral administrations of each amoxicillin formulation.

mg/5 ml and 500 mg AUC_{last}, respectively) were calculated by applying the linear trapezoid rule. Extrapolation of these areas to infinity ($AUC_{0-\infty}$) was done by adding the value C12/ke to the calculated AUC_{last} (where C12 = plasma concentration calculated from the log-linear regression equation obtained for the estimation of ke 12 h after dose).

Results

Each amoxicillin formulation was well tolerated at the administered doses. One male volunteer presented with substantial increases in AST, ALT and γ-GT levels on post-study evaluation, but these changes were considered not related to amoxicillin administration. All other biochemical parameters showed no clinically relevant alterations. No adverse effects were reported or observed.

The collision-induced dissociation (CID) of amoxicillin (m/z 366) showed a 349.2 daughter dissociated molecule and the CID of cefadroxil (m/z 364) showed a 207.8 daughter. The selected ion spectrograms with respective molecular protonated ions for amoxicillin and cefadroxil are shown in Figure 1. Typical selected ion chromatogram (MRM) plots are shown in Figure 2. The calibration curve of amoxicillin is illustrated in Figure 3 and the calculated linearity was 0.99 ± 0.004 (mean ± SDM, n = 5).

The mean absolute recovery of amoxicillin in plasma was 52.0% at 0.1 µg/ml and 65.0% at 5.0 µg/ml whereas for cefadroxil was 40.0% at 0.2 µg/ml. The limit of quantification was 0.05 µg/ml.

Four quality controls (0.15, 1, 4 and 12 µg/ml) were chosen. The intra-day variability was 6.0, 5.7, 1.8 and 1.7%, respectively, and the inter-day variability was 4.9, 8.0, 4.2 and 7.2%, respectively.

Amoxicillin peak plasma concentrations observed were 5.31 and 4.94 µg/ml for 250 mg/5 ml of oral suspension of Amoxicilina and Amoxil, respectively, and 7.11 and 7.73 µg/ml for 500 mg (capsules) of Amoxicilina and Amoxil, respectively.

The amoxicillin mean plasma concentrations vs. time profile obtained after the single oral administrations of each amoxicillin 250 mg/5 ml oral suspension and 500 mg capsule formulation are shown in Figure 4.

Table 1. Mean pharmacokinetic parameters obtained in 24 volunteers after administration of each 250 mg/5 ml amoxicillin oral suspension.

Parameter	Amoxicilina 250 mg/5 ml	Amoxil 250 mg/5 ml
AUC_{last} ((µg×h)/ml)		
Geom. Mean	12.44	12.05
90% CI	7.69 – 17.25	7.50 – 17.56
$AUC_{0-\infty}$ ((µg×h)/ml)		
Geom. Mean	12.67	12.32
90% CI	7.87 – 17.51	7.73 – 17.73
AUC_{all} (0 – 8 h) ((µg×h)/ml)		
Geom. Mean	12.49	12.11
90% CI	7.83 – 17.41	7.50 – 17.56
C_{max} (µg/ml)		
Geom. Mean	5.31	4.94
90% CI	2.44 – 9.82	2.49 – 8.37
Ke (h^{-1})		
Geom. Mean	0.61	0.63
90% CI	0.34 – 0.83	0.39 – 0.92
$t_{1/2}$ (h)		
Geom. Mean	1.2	1.2
90% CI	0.8 – 2.1	0.8 – 1.8
t_{max} (h)		
Median	1.25	1.0
Range	0.5 – 2.0	0.5 – 2.0

Table 2. Mean pharmacokinetic parameters obtained from 24 volunteers after administration of each 500 mg amoxicillin capsule formulation.

Parameter	Amoxicilina 500 mg	Amoxil 500 mg
AUC_{last} ((µg×h)/ml)		
Geom. Mean	20.83	22.06
90% CI	10.92 – 38.16	12.13 – 33.80
$AUC_{0-\infty}$ ((µg×h)/ml)		
Geom. Mean	21.01	22.26
90% CI	11.08 – 38.29	12.25 – 33.94
AUC_{all} (0 – 12 h) ((µg×h)/ml)		
Geom. Mean	20.91	22.16
90% CI	11.02 – 38.16	12.21 – 33.80
C_{max} (µg/ml)		
Geom. Mean	7.11	7.73
90% CI	3.11 – 14.78	3.26 – 14.98
Ke (h^{-1})		
Geom. Mean	0.58	0.60
90% CI	0.38 – 0.88	0.44 – 0.98
$t_{1/2}$ (h)		
Geom. Mean	1.2	1.2
90% CI	0.8 – 1.8	0.7 – 1.6
t_{max} (h)		
Median	2.0	1.75
Range	1.0 – 4.0	1.0 – 4.0

Tables 1 and 2 show the mean pharmacokinetic parameters obtained from 24 volunteers after administration of each 250 mg/5 ml and 500 mg amoxicillin oral formulations. Table 3 presents the ratios and the respective confidence intervals for bioequivalence analysis.

Discussion

The LC-MS-MS method described and used here for drug quantification provides the high sensitivity, specificity and high sample throughput required for pharmacokinetic studies [Bennett et al. 1997]. Amoxicillin plasma levels have been detected by other methods such as high pressure liquid chromatography coupled to UV detection [Muth et al. 1996] and by column switching ion-pair reversed phase liquid chromatographic systems with post-column derivatization [Carlqvist and Westerlund 1985]. In the former, the limit of quantitation was similar to ours (50 ng/ml), however, the elution times for both amoxicillin and cefadroxil (31.8 and 32.8 min, respectively) were 10 times longer than in our method. In the latter method, although the limit of quantitation was 10 ng/ml, no internal standard was used, the chromatography was more complex due to post-column derivatization, and the retention time was also longer for the analyte (approximately 10 min). Derivatization of amoxicillin to a fluorescent compound presented similar sensitivity and slightly longer retention time (4.5 min for amoxicillin), but it does involve a complex chromatography system including electrochemical detection [Mascher and Kikuta 1990]. With the experimental conditions used by us, the collision-induced dissociation (CID) of amoxicillin (m/z 366) showed a fragment at m/z 349.2 and the CID of cefadroxil (m/z 364) showed a fragment at m/z 207.8, similar to those described elsewhere [Rourick et al. 1996, Straub and Voyksner 1993, Suwanrumpha et al. 1988]. The daughter of the amoxicillin molecule (m/z 349) can be explained by the loss of a NH_3 at the amino side chain [Straub and Voyksner 1993, Suwanrumpha et al. 1988] whereas the daughter of cefadroxil (m/z 208) is probably due to cleavage of the lactam substructure [Rourick et al. 1996].

Amoxicillin peak plasma concentrations for both oral formulations and the t_{max} were similar to those reported in the literature [PDR 1999]. As expected, the capsule for-

Table 3. Geometric mean of the individual AUC_{last}, $AUC_{0-\infty}$, and C_{max} ratios (test/reference formulation) and the respective 90% confidence intervals (CI).

Amoxicilina/Amoxil	Statistical analysis			
	250 mg/5 ml		500 mg	
	Geom. mean	90% CI	Geom. mean	90% CI
AUC_{last} % ratio	103.70	97.82 – 109.94	93.26	85.0 – 102.33
$AUC_{0-\infty}$ % ratio	103.15	97.40 – 109.24	93.27	85.12 – 102.31
C_{max} % ratio	106.79	96.38 – 118.33	90.74	80.14 – 102.73

mulation (500 mg) presented higher C_{max}, later t_{max} and larger AUC compared to the oral suspension formulation (250 mg/5 ml).

Since the 90% CI for $AUC_{(0-8h)}$ and $AUC_{(0-12h)}$ mean ratios are within the 80 to 125% interval proposed by the US Food and Drug Administration authorities [1993, 1998], it is concluded that both Amoxicilina formulations (250 mg/5 ml oral suspension and 500 mg capsule) are bioequivalent to Amoxil 250 mg/5 ml and to Amoxil 500 mg, respectively, in both extent and rate of absorption.

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SHORT COMMUNICATION

Comparative Bioavailability of Two Cefadroxil Formulations in Healthy Human Volunteers After a Single-Dose Administration

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ABSTRACT: *Objective.* To compare the bioavailability of two cefadroxil capsule (500 mg) formulations (Cefadroxila from Eurofarma Laboratórios Ltd, Brazil, as test formulation and Cefamox® from Bristol–Myers Squibb, Brazil S.A. as reference formulation) in 24 volunteers of both sexes.

Material and methods. The study was conducted open with randomized two-period crossover design and a 1-week washout period. Plasma samples were obtained over a 12-h interval. Cefadroxil concentrations were analysed by combined reversed-phase liquid chromatography and tandem mass spectrometry (LC-MS-MS) with positive ion electrospray ionization using a selected ion monitoring method. From the cefadroxil plasma concentration versus time curves the following pharmacokinetic parameters were obtained: AUC_{last}, $AUC_{0-\infty}$ and C_{max}.

Results. Geometric mean of Cefadroxila/Cefamox® 500 mg individual percent ratio was 103.97% for AUC_{last}, 104.08% for $AUC_{0-\infty}$ and 95.23% for C_{max}. The 90% confidence intervals (CI) were 98.14–110.16%, 98.37–110.12%, and 85.59–105.96%, respectively.

Conclusion. Since the 90% CI for C_{max}, AUC_{last} and $AUC_{0-\infty}$ were within the 80–125% interval proposed by the Food and Drug Administration, it was concluded that the Cefadroxila 500 mg capsule was bioequivalent to the Cefamox® 500 mg capsule, according to both the rate and extent of absorption.

Key words: cefadroxil; bioavailability; mass spectrometer; chromatography

Introduction

Cefadroxil monohydrate {5-Thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylic acid,7-[[amino(4-hydroxyphenyl)acetyl]amino] - 3 - methyl - 8 - oxo-monohydrate, [6R[6a,7b(R*)]]} is a semisynthetic cephalosporin antibiotic intended for oral administration. It is a white to yellowish–white crystalline powder. It has the molecular weight of 381.40 kDa and it is commercially available in capsules (500 mg), tablets (1.0 g) and oral suspensions.

The objective of this study was to evaluate, in healthy volunteers, the bioequivalence of a test formulation of the 500 mg (capsule) of cefadroxil, elaborated by Eurofarma Laboratórios Ltd, Brazil (Cefadroxila) and a commercial formulation of 500 mg (capsule) of cefadroxil (Cefamox®) made by Bristol–Myers Squibb Brasil S.A. employed as reference formulation.

Methods

Clinical Protocol

Twenty-four healthy volunteers of both sexes aged between 18 and 50 years old and within the

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15% of the ideal body weight were selected for the study. The male group was composed of 12 volunteers (32.0 ± 7.0 years, mean ± S.D.; range 23–43 years), height between 160 and 180 cm (170.3 ± 5.7 cm), weighing between 56.7 and 97.6 kg (78.7 ± 13.2 kg). The female group was also composed of 12 volunteers (32.2 ± 6.1 years; range: 21–42 years), height between 152 and 167 cm (156.1 ± 5.0 cm), weighing between 43.0 and 67.5 kg (58.2 ± 7.1 kg).

All subjects gave written informed consent and the Institute of Biomedical Sciences of the University of Sao Paulo ethics committee approved the clinical protocol. All volunteers were healthy as assessed by physical examination, ECG, and the following laboratory tests: blood glucose, urea, creatinine, AST, ALT, alkaline phospatase, γ-GT, total bilirubin, albumin and total protein, trygliceride, total cholesterol, haemoglobin, haematocrit, total and differential white cell counts, and routine urinalysis. All subjects were negative for HIV, HBV (except for serological scar) and HCV.

The study was conducted in an open randomized two-period crossover balanced design with a 1-week washout period between the doses. During each period, the volunteers were hospitalized at 11:00 p.m. having already had a normal evening meal, and after an overnight fast they received at 6:00 a.m. a single 500 mg cefadroxil capsule of either laboratory. Water (200 mL) was given immediately after drug administration. All volunteers were then fasted for 4 h following the drug administration, after which a standard lunch was consumed and an evening meal was provided 10 h after dosing. No other food was permitted during the 'in-house' period. Liquid consumption was permitted *ad libitum* after lunch but xanthine-containing drinks including tea, coffee, and cola were avoided.

Systolic and diastolic arterial pressure (measured non-invasively with a sphygmomanometer), heart rate and temperature were recorded just before and hourly after drug administration.

Formulations

The following formulations were employed: Cefadroxila 500 mg capsules(lot number PI 045/00, expiration date 05/2002) as test formulation, and Cefamox® 500 mg capsules (lot number 133982, expiration date 11/2001) as reference formulation.

Drug Analysis

Blood samples (8 mL) from a suitable antecubital vein were collected into EDTA containing tubes before and 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 4, 6, 8, 10 and 12 h after the administration of each cefadroxil capsule formulation (500 mg). The blood samples were centrifuged at 3500 rpm for 10 min at room temperature and the plasma decanted and stored at −20°C until assayed for their cefadroxil content. All samples from a single volunteer were analysed on the same day in order to avoid interassay variation. Cefadroxil and the internal standard amoxicillin were extracted from volunteers' plasma by solid-phase extraction using Oasis cartridges. The compounds were eluted with a small volume of mobile phase, an aliquot of which was analysed by combined reversed phase liquid chromatography tandem mass spectrometry with positive ion electrospray ionization using selected daughter ion monitoring (multiple reaction mode (MRM)).

Briefly, 0.5 mL of purified water was added to a glass tube, followed by the plasma sample (50 µL) and internal standard (50 µL of amoxicillin, 1 µg/mL). The tube was vortex-mixed for 10 s and allowed to stand at room temperature for 5 min. Oasis SPE cartridges were pre-conditioned with methanol (1 mL) followed by purified water (1 mL), followed by the sample application (1:40). After two 2-mL water washings, the column was dried by vacuum suction and the cartridges were washed with acetonitrile 50% + 10 mM formic acid (2 mL, at a flow rate lower than 1 mL/min) and an aliquot (200 µL) was transferred to microvials

Chromatographic Conditions

Chromatography was performed on a Genesis C_{18} 4 µm analytical column (150 mm × 4.6 mm i.d.) fitted with a guard column of the same material. Chromatography was performed isocratically using a mobile phase composed of 50% acetonitrile containing 10 mM formic acid at a flow rate of 0.5 mL/min. The pressure of the

system was about 70–90 bar with a new guard column in place. The column was operated at a temperature of 26°C and a column switch was employed to divert column eluant to waste at appropriate times. A split of the column eluant of approximately 1:10 was included so that only approximately 50 µL/min entered the mass spectrometer. The temperature of the autosampler was maintained at 5°C and the injection volume was 20 µL.

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The drug analysis data were acquired and elaborated with MassLynx (v. 3.2) running under Windows NT (v. 4.0) on a Pentium PC. The total run time was set for 4.0 min and the typical standard retention times were 2.57 min for both cefadroxil and the internal standard amoxicillin. The mass spectrometer (Micromass model Quattro LC) equipped with electrospray source using a crossflow counter electrode) was run in positive mode (ES+) and set with a MRM of the following ions 364.0 > 207.8 and 366.0 > 349.2 for cefadroxil and amoxicillin, respectively. For cefadroxil and amoxicillin the dwelling time, the cone voltage, the collision energy, and gas pressure (Argon) were 0.5 s, 20.2 V, 10 eV and 3×10^{-3} bar, respectively.

Pharmacokinetics and Statistical Analysis

The first-order terminal elimination rate constant (k_e) was estimated by linear regression from the points describing the elimination phase on a log–linear plot. Half-life ($t_{1/2}$) was derived from this rate constant ($t_{1/2} = \ln(2)/k_e$). The maximum observed plasma concentration (C_{max}) and the time taken to achieve this concentration (T_{max}) were obtained directly from the curves. The areas under the cefadroxil plasma concentration versus time curves from 0–12 h (AUC_{all}) and from 0 to the last detectable concentration (AUC_{last}) was calculated by applying the linear trapezoid rule. Extrapolation of these areas to infinity ($AUC_{0-\infty}$) was done by adding the value C_{last}/k_e to the calculated AUC_{last} (where C_{last} = the last detectable concentration).

Results

One male volunteer dropped out of the study because he presented fever before the drug administration in the second confinement period. Each cefadroxil formulation was well tolerated at the administered doses. A volunteer refereed mild nausea not related to the drug. All biochemical parameters did not present any clinical relevant alterations. No other adverse effects were reported or observed.

The mean absolute recovery of cefadroxil in plasma was 26.4% at 0.15 µg/mL and 16.3% at 14.0 µg/mL whereas for amoxicillin was 28.1% at 1.0 µg/mL. The limit of quantification was 0.05 µg/mL.

Five qualities controls (0.15, 1, 4, 15 and 27 µg/mL) were chosen. The intra-day precision was 10.2, 10.3, 8.7, 4.6 and 3.0%, respectively, and the intra-day accuracy was 98.1, 112.5, 112.0, 113.2 and 109.7%, respectively. The inter-day precision was 3.1, 6.4, 8.1, 13.0 and 0.6, respectively, and the inter-day accuracy was 94.7, 108.1, 108.1, 104.2 and 110.4%, respectively.

Mass Spectrum

The collision-induced dissociation (CID) of cefadroxil (m/z 364) showed a 207.8 daughter dissociated molecule and the CID of amoxicillin (m/z 366) showed a 349.2 daughter. A typical selected ion chromatogram (MRM) plot is shown in Figure 1.

The cefadroxil mean plasma concentrations versus time profile obtained after the single oral administration of the 500 mg capsule formulation are shown in Figure 2.

Table 1 shows the mean pharmacokinetic parameters obtained from 23 volunteers after administration of the 500 mg cefadroxil oral formulation (capsule). Table 2 presents the ratios and the respective 90% confidence intervals (CI) for bioequivalence analysis.

Discussion

The LC-MS-MS method described here for drug quantification agrees with the concepts of high sensitivity, specificity and high samples

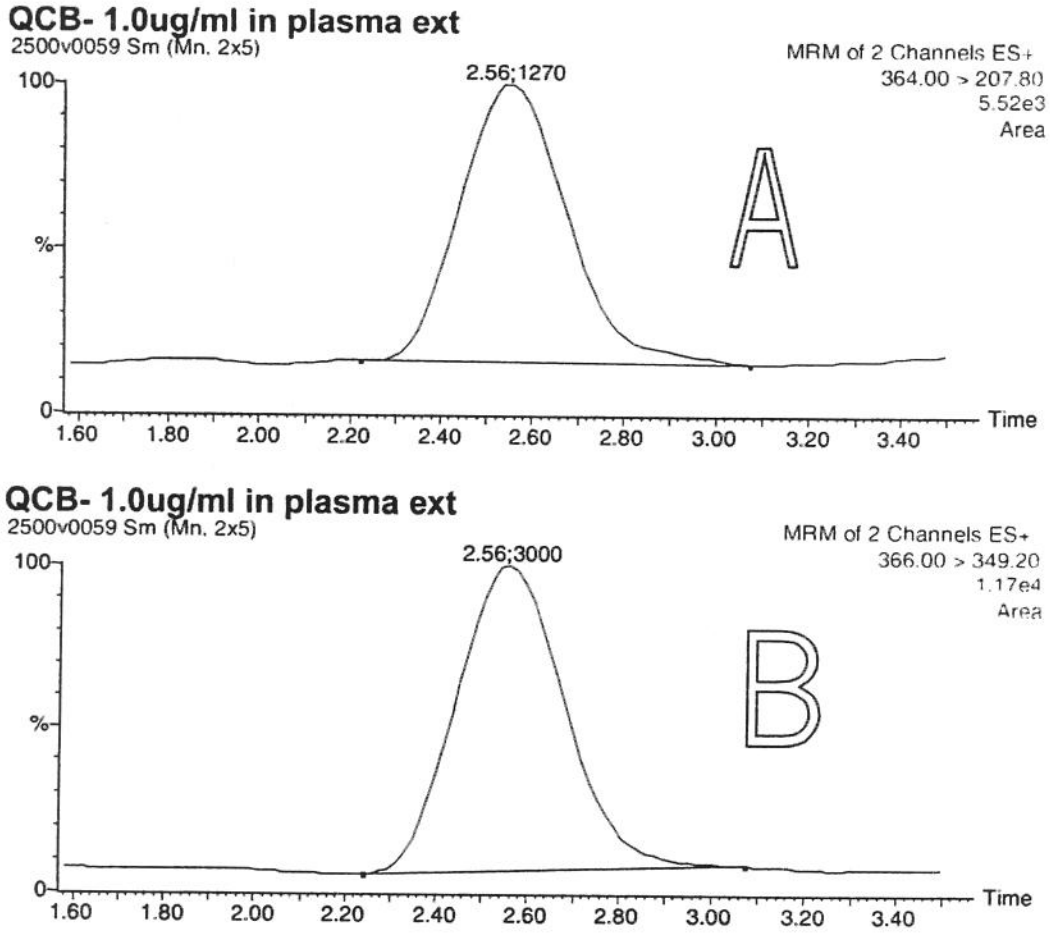

Figure 1. Selected ion chromatograms (MRM). A: MRM of two channels ES + : 364.00 > 207.80 for cefadroxil. B: MRM of two channels ES + : 360.00 > 349.20 for amoxicillin. The peaks illustrate the retention time and integrated area

throughput required for pharmacokinetic studies [1]. Cefadroxil levels in either plasma or serum have been detected by other methods such as bioassay [2] or liquid chromatography coupled to UV detection [3]. Although the latter technique is currently used for drug quantitation in biological fluids, the elution time for both cefadroxil and amoxicillin (between 31.8 and 32.8 min for cefadroxil and 34.8 and 38.8 for amoxicillin) were ten times longer than in our method (2.57 min for both drugs) [3]. Nowadays bioassay is rarely employed for drug quantitation, mainly due to the unespecificity of this method.

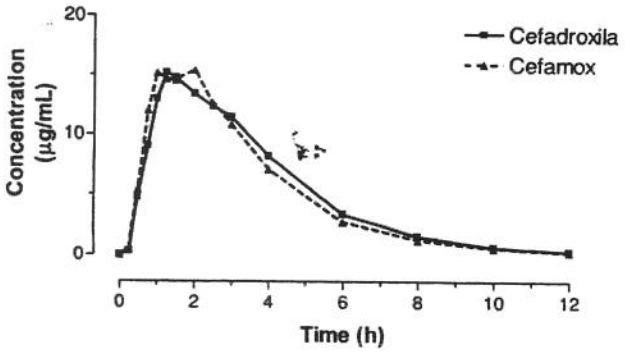

Figure 2. Cefadroxil plasma mean concentrations versus time profile obtained after the single oral administration of 500 mg of the cefadroxil capsule formulation

In our experimental conditions, the CID of cefadroxil (m/z 364) showed a fragment at m/z 207.8 and the CID of amoxicillin (m/z 366) showed a fragment at m/z 349.2, similar to those described elsewhere [4–6].

Cefadroxil peak plasma concentrations of both oral formulations and the T_{max} were similar to those reported in the literature [2,7].

Table 1. Mean pharmacokinetic parameters obtained from 23 volunteers after administration of each 500 mg cefadroxil capsule formulation

Parameter	Cefadroxila 500 mg	Cefamox® 500 mg
AUC_{last} ([μg · h]/mL)		
Geometric mean	61.42	59.56
90% CI	33.96–82.18	33.80–90.22
$AUC_{0-\infty}$ ([μg · h]/mL)		
Geometric mean	62.51	60.60
90% CI	35.61–83.42	34.40–91.21
AUC_{all} (0–12 h) ([μg · h]/mL)		
Geometric mean	61.48	59.67
90% CI	33.96–82.18	34.13–90.22
C_{max} (μg/L)		
Geometric mean	19.34	20.64
90% CI	8.74–35.75	8.46–43.32
k_e (h^{-1})		
Geometric mean	0.40	0.40
90% CI	0.24–0.52	0.23–0.55
$T_{1/2}$ (h)		
Geometric mean	1.8	1.9
90% CI	1.3–2.8	1.3–3.0
T_{max} (h)		
Geometric mean	1.5	1.3
90% CI	1.0–4.0	0.8–3.0

Table 2. Geometric mean of the individual AUC_{last}, $AUC_{0-\infty}$, and C_{max} ratios (test/reference formulation) and the respective 90% confidence intervals (CI)

Cefadroxila/Cefamox® 500 mg	Statistical analysis	
	Geometric mean (%)	90% CI
AUC_{last}% ratio	103.97	98.14–110.16
$AUC_{0-\infty}$% ratio	104.08	98.37–110.12
C_{max}% ratio	95.23	85.59–105.96

Since the 90% CI for C_{max}, AUC_{last} and $AUC_{0-\infty}$ mean ratios are within the 80–125% interval proposed by the US Food and Drug Administration [8,9], it is concluded that the Cefadroxila formulation tested elaborated by Eurofarma Laboratórios Ltd, Brazil (500 mg capsule) is bioequivalent to Cefamox® 500 mg, for both extent and rate of absorption.

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Terbinafine Quantification in Human Plasma by High-Performance Liquid Chromatography Coupled to Electrospray Tandem Mass Spectrometry: Application to a Bioequivalence Study

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Summary: A method based on liquid chromatography with positive ion electrospray ionization and tandem mass spectrometry is described for the determination of terbinafine in human plasma using naftifine as internal standard. The method has a chromatographic run time of 5 minutes and was linear in the range 1.0 to 2000 ng/mL. The limit of quantification was 1.0 ng/mL; the intraday precision was 3.6%, 3.8%, 3.5%, and 4.1%; and the intraday accuracy was −2.7%, 7.7%, 4.8%, and −2.7% for 5.0, 80.0, 250.0, and 1500.0 ng/mL, respectively. The interday precision was 4.9%, 1.7%, 2.4%, and 4.6% and the interday accuracy was 0.3%, 5.8%, 6.5%, and −1.4% for the same concentrations. This method was used in a bioequivalence study of two tablet formulations of terbinafine. Twenty-four healthy volunteers (both sexes) received a single oral dose of terbinafine (250 mg) in an open, randomized, two-period crossover study. The 90% CI of geometric mean ratios between (Terbinafina®; Medley S/A Indústria Farmacêutica, Campinas, Brazil) and Lamisil® (Novartis Biociências S/A, São Paulo, Brazil) were 90.5% to 110.0% for C_{max}, 92.2% to 108.1% for AUC_{last}, and 91.3% to 107.5% for AUC_{0-inf}. Because the 90% CI for the above-mentioned parameters were included in the 80% to 125% interval proposed by the US FDA, the two formulations were considered bioequivalent in terms of rate and extent of absorption. **Key Words:** LC-MS-MS—HPLC—Naftifine—Lamisil—Terbinafine.

Terbinafine is a synthetic allylamine antifungal compound. Chemically, terbinafine is (*E*)-N-(6,6-dimethyl-2-hepten-4-ynyl)-N-methyl-1-naphthalene methanamine. The empirical formula is $C_{21}H_{25}N$ with molecular weight of 291.4 g/mol. Terbinafine is hypothesized to act by inhibiting squalene epoxidase, thus blocking the biosynthesis of ergosterol, an essential component of fungal cell membranes (1–2).

Terbinafine concentrations in human plasma and other tissues have already been detected (3–7). Most studies use high-performance liquid chromatography (HPLC) with ultraviolet detection (UV) to determine terbinafine concentrations. Besides its high sensitivity, this assay is usually slower than other methods, such as HPLC coupled to tandem mass spectrometry (LC-MS-MS). In this work, we describe a fast, sensitive, and specific LC-MS-MS method for the quantitation of terbinafine using naftifine as internal standard. The method was applied to a study of bioequivalence of two oral formulations of terbinafine: 250 mg tablet (Terbinafina®; Medley S/A Indústria Farmacêutica, Campinas, Brazil) as test formulation and Lamisil® (Novartis Biociências S/A, São Paulo, Brazil) as reference formulation).

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MATERIALS AND METHODS

Chemicals and Reagents

Terbinafine hydrochloride (lot number HX0103) was provided by Medley, Campinas, Brazil. Naftifine hydrochloride (lot number FCZ01) was obtained from TCI America (Portland, OR). Acetonitrile and methanol, HPLC-grade; and hexanes (analytical-grade) were purchased from Mallinckrodt (Paris, KY). Formic acid (analytical-grade) was purchased from Merck (Rio de Janeiro, Brazil). Ultra-pure water was obtained from an Elga UHQ system (High Wycombe, Bucks, UK). Blank human blood was collected from healthy, drug-free volunteers. Plasma was obtained by centrifuging blood treated with the anticoagulant sodium heparin. Pooled plasma was prepared and stored at approximately −20°C until needed.

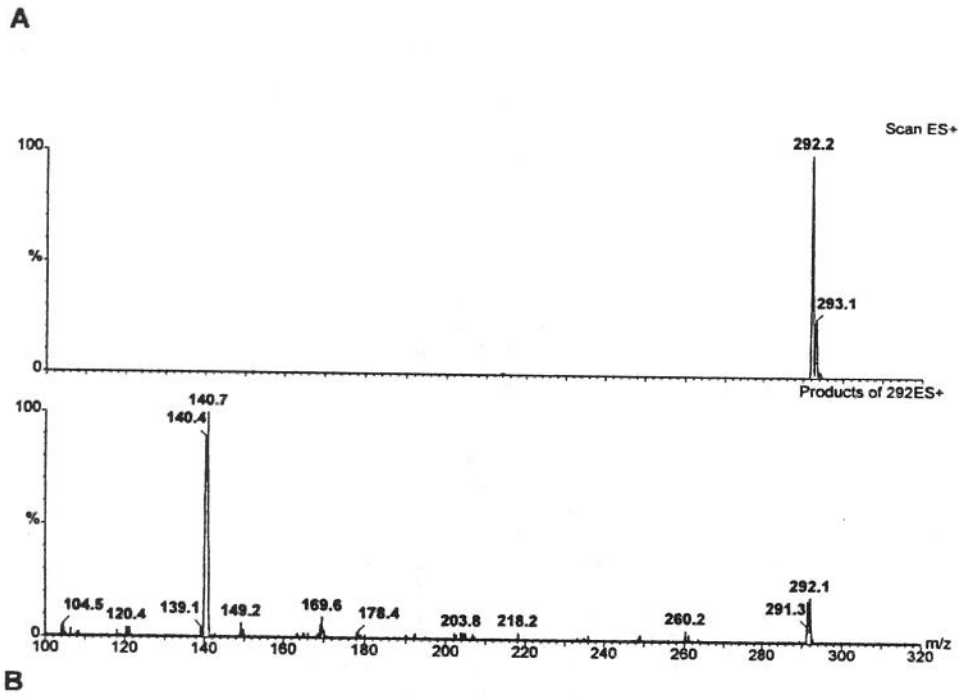


FIG. 1. Full scan mass spectra and respective product ion spectra of (A) terbinafine and (B) naftifine.

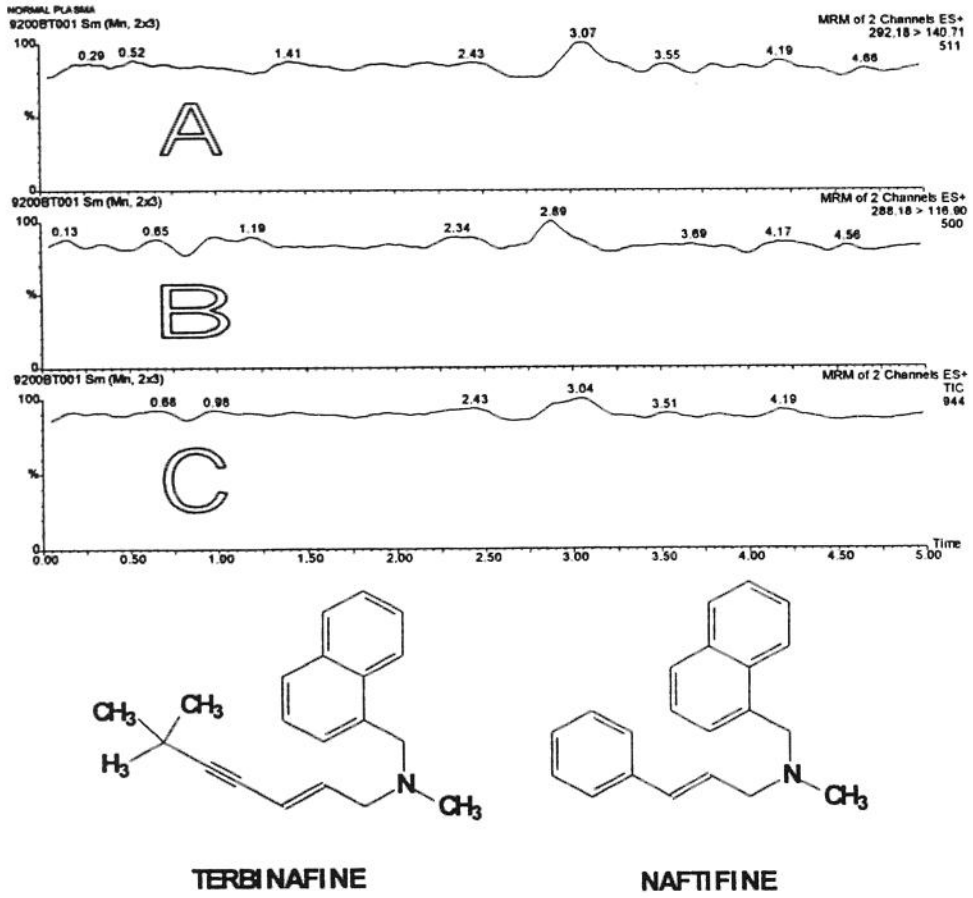
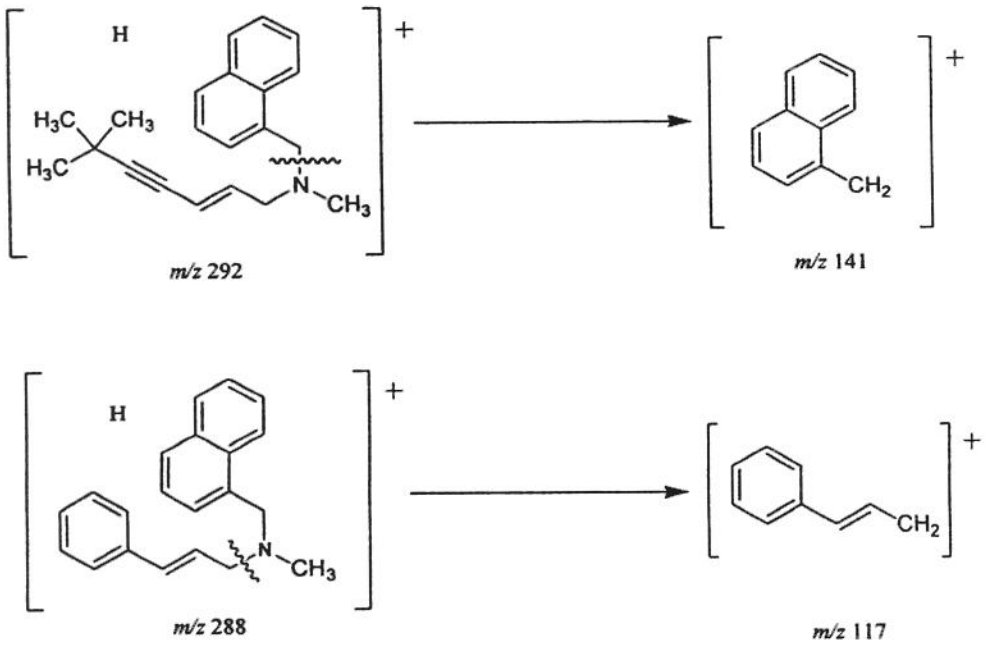


FIG. 2. MRM chromatograms of blank human plasma: (A) terbinafine, (B) naftifine, and (C) TIC. Dissociation route proposed for terbinafine and naftifine.



Calibration Standards and Quality Control

Stock solutions of terbinafine and IS were prepared in methanol–water (50:50 v/v) at concentrations of 1 mg/mL. Calibration curves of terbinafine were prepared in blank plasma at concentrations of 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0, 500.0, 1000.0, and 2000.0 ng/mL and performed in duplicate for each batch. The quality control samples were prepared in blank plasma at concentrations of 5.0, 80.0, 250.0, and 1500.0 ng/mL (QCA, QCB, QCC, and QCD, respectively).

Sample Preparation

All frozen human plasma samples were previously thawed at ambient temperature and centrifuged at approximately 4550*g* for 5 minutes at 4°C to precipitate solids. Twenty μL internal standard solution (1μg/mL naftifine in 50:50 v/v methanol:water solution) were added to a 200-μL aliquot of plasma sample. The tubes were briefly vortex-mixed and the compounds of interest were extracted with 4 mL diethyl ether:hexanes (80:20 v/v). The mixture was vortex-mixed for approximately 40 seconds, and the organic phase was evaporated under N_2 at 37°C. The dry residues were reconstituted with 200 μL mobile phase (80% CH_3CN, 20% H_2O containing 10 mmol/L formic acid) and transferred to the autoinjector microvials.

Chromatographic Conditions

An aliquot (20 μL) of each plasma extract was injected into a Genesis C_{18} 4-μm analytical column (150 mm × 4.6 mm i.d.) operated at a temperature of 40°C. The compounds were eluted by pumping the mobile phase at a flow rate of 0.5 mL/min. Under these conditions, typical standard retention times were 2.8 minutes for terbinafine and 2.9 minutes for naftifine, and back-pressure values of approximately 50 to 70 bar were observed.

A split of the column eluant of approximately 1:10 was included so that only 50 μL/min entered to the mass spectrometer. The temperature of the autosampler was kept at 5°C and the run-time was 5.0 minutes.

Mass-Spectrometric Conditions

The Quattro II (Micromass, Altringham, Cheshire, UK) mass spectrometer equipped with an electrospray source using a crossflow counter electrode was run in positive mode (ES+), and set up in multiple reaction mode (MRM), monitoring the transitions 292.2 > 140.7 and 288.2 > 116.9, for terbinafine and naftifine, respectively (full- scan spectra are illustrated in Figure 1A and 1B). For both terbinafine and naftifine the dwell time and the collision gas pressure (argon) were 0.5 seconds and 1.0×10^{-3} mBar, respectively. The cone voltage and the collision energy were 35 V and 15 eV for terbinafine and 25V and 12 eV for naftifine, respectively. Data acquisition and analysis were performed using the MassLynx (Micromass; Wythenshawe, Manchester, UK) (v 3.2) software running under Windows NT (v 4.0) (Microsoft; Redmond, WA) on a Digital Celebris GL 6200 PC (Compaq Computer; Reading, Berks, UK).

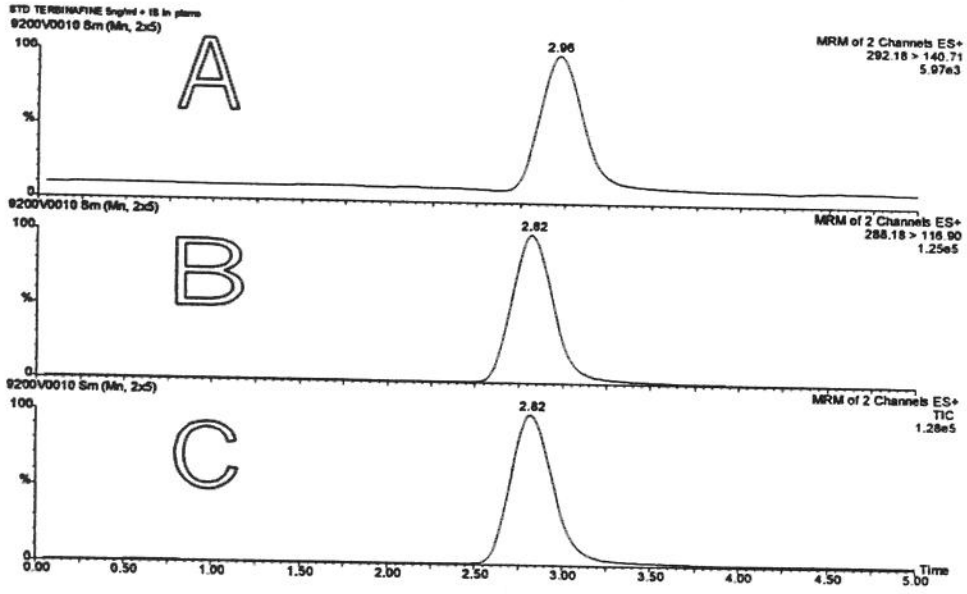


FIG. 3. MRM standards chromatograms: (**A**) terbinafine and (**B**) naftifine, and (**C**) TIC.

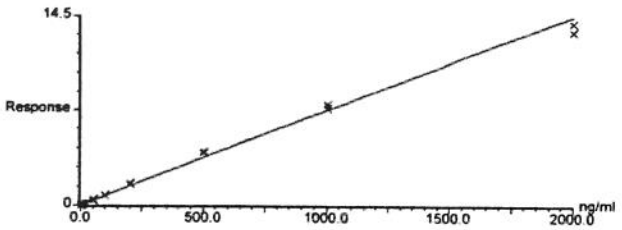


FIG. 4. Calibration curve of terbinafine.

Bioequivalence Study

This method was applied to evaluate the bioequivalence of two tablet formulations of terbinafine in healthy volunteers: Terbinafina® (test formulation from Medley S.A. Indústria Farmacêutica, Brazil; lot number Teb 06/00–6, expiration date Jun/2002) and Lamisil® (standard reference formulation from Novartis Biociências S.A., Brazil; lot number Z65836, expiration date Jun/2002). Bioequivalence between the two formulations was assessed by calculating individual test/reference ratios for the peak of concentration (C_{max}), area under the plasma concentration curve (AUC) until the last observed concentration (AUC_{last}), and the area under the curve between the first sample (pre-dosage) and infinity (AUC_{0-inf}), along with their means and 90% confidence intervals (CI) after logarithmic transformation of the data (additive model). The inclusion of the 90% CI of the ratios into the 80% to 125% interval was analyzed by ANOVA.

Twenty-four healthy volunteers of both sexes (12 men, 12 women), aged between 18 and 50 years old and within the 15% of the ideal body weight, were selected for the study after assessment of their health status by clinical evaluation (physical examination, ECG) and the following laboratory tests: blood glucose, urea, creatinine, uric acid, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase, γ-GT, total bilirubin, albumin and total protein, triglyceride, total cholesterol, hemoglobin, hematocrit, total and differential white cell counts, and routine urinalysis. All subjects were negative for human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV) (except for serologic scar).

The volunteers had the following clinical characteristics (divided by gender and expressed as mean ± SD [range]):

Men: age: 22.2 ± 3.7 years. [18–32], height: 175.0 ± 7.8 cm [163.5–185.0], body weight: 72.7 ± 14.7 kg [51.3–93.7].

TABLE 1. *Quantified concentration of individual samples for between-batch and within-batch validation**

Sample	QCA	QCB	QCC	QCD
Between batches				
Nominal concentration (ng/mL)	5.0	80	250	1500
Mean	4.9	86	262	1459
SD (n = 8)	0.2	3.3	9	60
Accuracy (%)	−2.7	7.7	4.8	−2.7
Precision (%)	3.6	3.8	3.5	4.1
Within batches				
Mean	5.0	85	266	1479
SD (n = 3)	0.2	1.5	6	68
Accuracy (%)	0.3	5.8	6.5	−1.4
Precision (%)	4.9	1.7	2.4	4.6

* Values given as ng/mL.

QCA, quality control A; QCB, quality control B; QCC, quality control C; QCD, quality control D; SD, standard deviation.

TABLE 2. *Mean pharmacokinetic parameters obtained from 24 volunteers after administration of 250-mg terbinafine tablet*

Parameter	Lamisil®*	Terbinafina®†
AUC_{last} ([ng · h]/mL)		
Geometric mean	5535	5642
SD	2153	2613
AUC_{0-inf} ([ng · h/mL)		
Geometric mean	5853	5931
SD	2369	2863
C_{max} (ng/L)		
Geometric mean	1094	1106
SD	415	458
$T_{1/2}$ (h)		
Geometric mean	41.75	34.95
Range	15.6–98.8	19.7–78.7
T_{max} (h)		
Geometric mean	2.0	1.5
Range	1.0–3.0	1.0–3.0

* Novartis Biociências S/A, Brazil.

† Medley S/A Indústria Farmacêutica, Brazil.

AUC_{last}, area under the plasma concentration curve until the last concentration observed; AUC_{0-inf}, area under the curve between the first sample and infinity; C_{max}, maximum concentration; SD, standard deviation; $T_{1/2}$, half-life; T_{max} time of occurrence for maximum (peak) drug concentration.

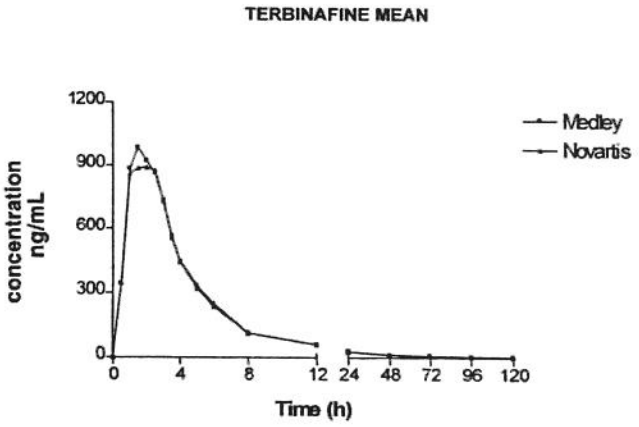


FIG. 5. Mean plasma concentrations versus time curve for both terbinafine formulations.

Women: age: 24.3 ± 5.1 year. [20–37], height: 163.0 ± 5.3 cm [154.5–169.0], body weight: 57.6 ± 5.1 kg [50.0–69.5].

The study was a single-dose, two-way randomized crossover design with a 2-week washout period between doses. During each period, the volunteers were hospitalized at 9:00 PM having already had a normal evening meal, and after an overnight fast they received (at 6:00 AM) a single dose of terbinafine (250 mg, either tablet formulation). Two hundred milliliters of water were given immediately after drug administration, and the volunteers then fasted for 4 hours, after which period a standard lunch was served; an evening meal was provided 12 hours after dosing. No other food was permitted during the in-house period, and liquid consumption was allowed ad libitum after lunch (with no xanthine-containing drinks, including tea, coffee, and cola).

Systolic and diastolic arterial pressure (measured non-invasively with a sphygmomanometer), heart rate, and temperature were recorded just before and hourly after the administration of each terbinafine dose. Blood samples (6 mL) from a suitable antecubital vein were collected into EDTA-containing tubes before and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12, 24, 48, 72, 96, and 120 hours post-dosing. The blood samples were centrifuged at approximately 2,000*g* for 10 minutes at room temperature and the plasma decanted and stored at −20°C until assayed for their terbinafine content. All samples from a single volunteer were analyzed on the same day to avoid interassay variations.

RESULTS

Full-scan positive-ion mass spectra of terbinafine and naftifine showed the protonated molecular species $[M+H]^+$ at *m/z* 292.2 and 288.2, respectively. The most abundant ion in the product ion spectra was at *m/z* 140.7 for terbinafine and 116.9 for naftifine (Fig. 1A and 1B). From these results, the mass spectrometer was set as follows: *m/z* 292.2 for terbinafine and *m/z* 288.2 for naftifine as the precursor ions and *m/z* 140.7 and 116.9 as their respective product ions. The structure of protonated molecules and neutral losses are shown in Scheme 01 proposing a dissociation route for terbinafine and naftifine.

Under the LC-MS-MS conditions described above, and as shown in Figure 2, no endogenous peak was observed in the mass chromatogram of blank plasma. The mass chromatograms of a sample are shown in Figure 3, in which the retention times of terbinafine and IS were 2.8 and 2.9 minutes, respectively. Mean absolute recovery was 66.5% and 58.9% (10.0 and 500.0 ng/mL, respectively) for terbinafine, and 73.4% for naftifine.

Assay Performance

Linearity, precision, and accuracy were assessed for this method. Linearity of MN calibration curve was proven for the range from 1.0 to 2000.0 ng/mL (Fig. 4).

The coefficient of correlation mean was greater than 0.994. The lower limit of quantification (LOQ), defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, was 1.0 ng/mL. Although sensitivity was good enough to qualify even lower values, measures were taken to guarantee an LOQ approximately 0.1% to 0.3% of the anticipated C_{max}.

Precision and accuracy of quality controls are shown in Table 1. Precision was determined as the percent relative standard deviation RSD% = 100(SD/M) and accuracy as the percent relative error, RE% = (E-T)(100/T),

TABLE 3. *Geometric mean of the individual AUC_{last}, AUC_{0-inf}, and C_{max} ratios (test/reference formulation) and the respective 90% CI*

	Statistical analysis			
	Parametric		Non-parametric	
Terbinafina®*/Lamisil®†	Geometric mean	90% CI	Geometric mean	90% CI
AUC_{last} % ratio	99.8%	92.2–108.1%	100.1%	92.6–109.4%
AUC_{0-inf} % ratio	99.0%	91.3–107.5%	100.4%	92.5–108.9%
C_{max} % ratio	99.8%	90.5–110.0%	99.5%	89.0–110.2%

* Medley S/A Indústria Farmacêutica, Brazil.

† Novartis Biociências S/A, Brazil.

AUC_{last}, area under the plasma concentration curve until the last concentration observed; AUC_{0-inf}, area under the curve between the first sample and infinity; C_{max}, maximum concentration; CI, confidence interval.

where M is the mean, SD is the standard deviation, T is theoretical concentration, and E is the experimentally determined concentration.

Four female volunteers complained of mild headache, two female volunteers complained of mild diarrhea, and one female volunteer complained of mild nausea. Also, another female volunteer complained of mild dizziness and another of mild abdominal pain. Furthermore, another female volunteer complained of dysmenorrhea. All biochemical parameters monitored presented no clinically relevant alterations.

Table 2 shows the mean pharmacokinetic parameters obtained after the administration of 250 mg terbinafine to 24 healthy volunteers. Mean plasma terbinafine concentrations versus time curve is shown in Figure 5, and the statistical analysis of the bioequivalence parameters and their confidence intervals is depicted in Table 3.

DISCUSSION

Terbinafine concentrations in human plasma and other tissues such as human nails, skin surface biopsies, and human hair have already been detected by HPLC (3–7). Zehender et al. (3) described an HPLC-UV assay using a solid-phase extraction for terbinafine determination in plasma. The limit of quantification was 20 ng/mL, and the retention time was longer than 30 minutes. Denouël et al. (4), also using an HPLC-UV for terbinafine plasma detection, showed a low limit of quantification (2 ng/mL) but the retention time was also longer (by 10 min). Although these authors also used a liquid–liquid extraction, an additional second step was required, making the method more complex than ours.

Tandem mass spectrometry has been recently reported for terbinafine quantification in human hair and human plasma (7,8). Brignol et al. (8) measured terbinafine levels in human plasma using liquid chromatography coupled to tandem mass spectrometry equipped with an atmospheric chemical ionization (APCI) interface. The molecular mass fragmentation for terbinafine was similar to our study. The lower limit of quantification (LLOQ, 0.07 ng/mL) was achieved by using APCI instead of ESI, and by using deuterium-labeled terbinafine (8). However, the chromatographic method used presented a longer retention time (4.3 min), and deuterium-labeled isotopes are seldom commercially available.

Terbinafine plasma recovery varies between different studies, ranging from 53.4% to 104% (3–5,7,8). Zehender et al. (3), using HPLC-UV with solid-phase extraction, observed 55% recovery, which was associated with the lipophilic characteristics of terbinafine (3). Dykes et al. (5) and Denouël et al. (4), also using HPLC-UV assays, observed similar recoveries of terbinafine (more than 80%). Furthermore, Brignol et al. (8), using a protein precipitation method and analyzing by LC-MS-MS, observed recoveries between 53.4% and 73.4% (8), similar to ours. Although the method described here shows relatively lower recovery values than those performed by HPLC-UV, this fact did not compromise either the accuracy or the precision of the assay.

It is known that the latency period for the development of terbinafine liver injury is approximately 4 to 6 weeks (9). Because terbinafine exposure AUC is increased 16% by terbinafine, it would be interesting to determine whether higher terbinafine plasma levels could be associated with liver injury in some patients.

After the oral administration of terbinafine tablets to the volunteers, the observed terbinafine peak plasma concentration (C_{max}) values and the time values required to reach peak concentration (T_{max}) were similar to those reported in the literature (4,10) and equivalent between the formulations. In addition, the calculated 90% CI for mean C_{max}, AUC_{last}, and AUC_{0-inf} Terbinafina®/Lamisil® individual ratios were within the 80% to 125% interval defined by the US Food and Drug Administration (11,12). Thus, it is concluded that the terbinafine test formulation (Terbinafina®) is bioequivalent in terms of both rate and extent of absorption to the reference formulation (Lamisil®).

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Quantification of methyldopa in human plasma by high-performance liquid chromatography–electrospray tandem mass spectrometry Application to a bioequivalence study

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Abstract

A method based on LC–MS–MS is described for the determination of methyldopa in human plasma using dopa-phenyl-D3 as the internal standard. The method has a chromatographic run time of 5.5 min and was linear in the range of 20–5000 ng/ml. The limit of quantitation was 20 ng/ml, the intra-day precisions were 7.3, 5.4 and 4.3% and the intra-day accuracies were −8.0, −1.3 and −2.0% for 30, 600 and 3000 ng/ml, respectively. The inter-day precisions were 7.7, 0.5 and 0.7% and the inter-day accuracies were 0.2, −1.1 and −2.3%, respectively, for the above concentrations. This method was employed in a bioequivalence study of two tablet formulations of methyldopa.

Keywords: Bioequivalence studies; Methyldopa

1. Introduction

Methyldopa, 3-hydroxy-α-methyl-L-tyrosine, is an antihypertensive agent that primarily acts within the central nervous system as an α-adrenergic agonist [1]. Methyldopa is taken up by adrenergic neurons, where it is decarboxylated and hydroxylated to form the false transmitter, α-methylnoradrenaline, which is less active than noradrenaline on α_1-receptors and thus less effective in causing vasoconstriction.

In this paper, we describe a fast, sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for the quantitation of methyldopa using dopa-phenyl-D3 as internal standard (I.S.). The method was applied to a bioequivalence study of two oral formulations of methyldopa (500 mg tablet; Legrand Metildopa from EMS Indústria Farmacêutica, Brazil, as test formulation and Aldomet from Prodome Química e Farmacêutica, Brazil as reference formulation).

2. Experimental

2.1. Chemicals and reagents

Methyldopa was provided by EMS, Brazil, lot No. 00110806. Dopa-phenyl-D3 was purchased from

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CDN Isotopes, Canada, lot No. V166P7. Acetonitrile, methanol, and dichloromethane (HPLC grade), perchloric acid and formic acid (analytical grade) were purchased from Mallinckrodt (USA). Fuming hydrochloric acid was purchased from Merck, Brazil. Ultra pure water was obtained from an Elga UHQ system (Elga, UK). Blank human blood was collected from healthy, drug-free volunteers. Plasma was obtained by centrifugation of blood treated with the anticoagulant sodium heparin. Pooled plasma was prepared and stored at approximately −20 °C until needed.

2.2. Calibration standards and quality control

Stock solutions of methyldopa and I.S. were prepared in methanol–water (50:50, v/v) containing 1 m*M* hydrochloric acid at concentration of 1 mg/ml. The solutions were protected from light by wrapping the container with aluminum foil. Calibration standards of methyldopa were prepared in blank plasma at concentrations of 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/ml and were assayed in duplicate in each batch. Calibration standards were prepared in bulk and dispensed in 1.5-ml aliquots into properly labeled Eppendorf tubes and stored at −20 °C until required for assay. The quality control samples were prepared in blank plasma at concentrations of 30, 600 and 3000 ng/ml (QCA, QCB, and QCC, respectively).

2.3. Sample preparation

Prior to assay, frozen human plasma samples were thawed at ambient temperature and centrifuged at 2000 *g* for 5 min at 4 °C to precipitate solids. In the following order, 200 μl of ultra pure water, 50 μl of the internal standard solution (10 μg/ml dopa-phenyl-D3 in methanol–water, 50:50, v/v, solution), and 50 μl of 10% (v/v) perchloric acid were added to each disposable glass tube (non-siliconised; 15 ml) containing a 200-μl aliquot of plasma sample. The tubes were briefly vortex-mixed and then lipophilic impurities were extracted with 4 ml of dichloromethane. The mixture was vortex-mixed for approximately 40 s. The tubes were centrifuged at 2000 *g* for 10 min and 200 μl of the aqueous (upper) phase was then transferred to the HPLC auto-injector microvials.

2.4. Chromatographic conditions

An aliquot (40 μl) of each plasma extract was injected into a Genesis C_{18} 4 μm analytical column (100 mm×2.1 mm I.D.) preceded by a pre-column of the same material (10 mm×2.1 mm I.D.) at a flow-rate of 0.15 ml/min of the mobile phase [CH_3CN–water (10:90)+10 m*M* formic acid]. The column operated at room temperature (22–27 °C). Under these conditions, typical standard retention times were 3.3 min for both methyldopa and I.S., and back-pressure values of approximately 30–40 bar were observed.

A split of the column eluent of approximately 1:10 was included so that only 15 μl/min entered the mass spectrometer. The temperature of the auto-sampler was kept at 5 °C and the total run time was 5.5 min.

2.5. Mass spectrometric conditions

The mass spectrometer (Micromass Model Quattro Ultima) equipped with an electrospray source using a crossflow counter electrode was run in positive mode (ES+) with multiple reaction monitoring (MRM). Full-scan positive-ion mass spectra of methyldopa and dopa-phenyl-D3 showed the protonated molecular species $[M+H]^+$ at *m/z* 212.1 and 201.2, respectively. The most abundant ion in the product ion spectra was at *m/z* 166.1 for methyldopa and 154.2 for I.S. From these results, the mass spectrometer was set as follows: *m/z* 212.1 for methyldopa and *m/z* 201.2 for I.S. as the precursor ions and *m/z* 166.1 and 154.2 as the respective product ions (Fig. 1A–D). The proposed fragmentation route for methyldopa and I.S. is shown in Scheme 1.

For both methyldopa and I.S. the dwell time and the capillary were 0.8 s and 3.8 kV. The optimum values for both cone voltage and the collision energy were 25 V and 15 eV for methyldopa and 15 V and 16 eV for I.S., respectively. Data acquisition and analysis were performed using the software MassLynx (v 3.5) running under Windows NT (v 4.0) on a Pentium personal computer.

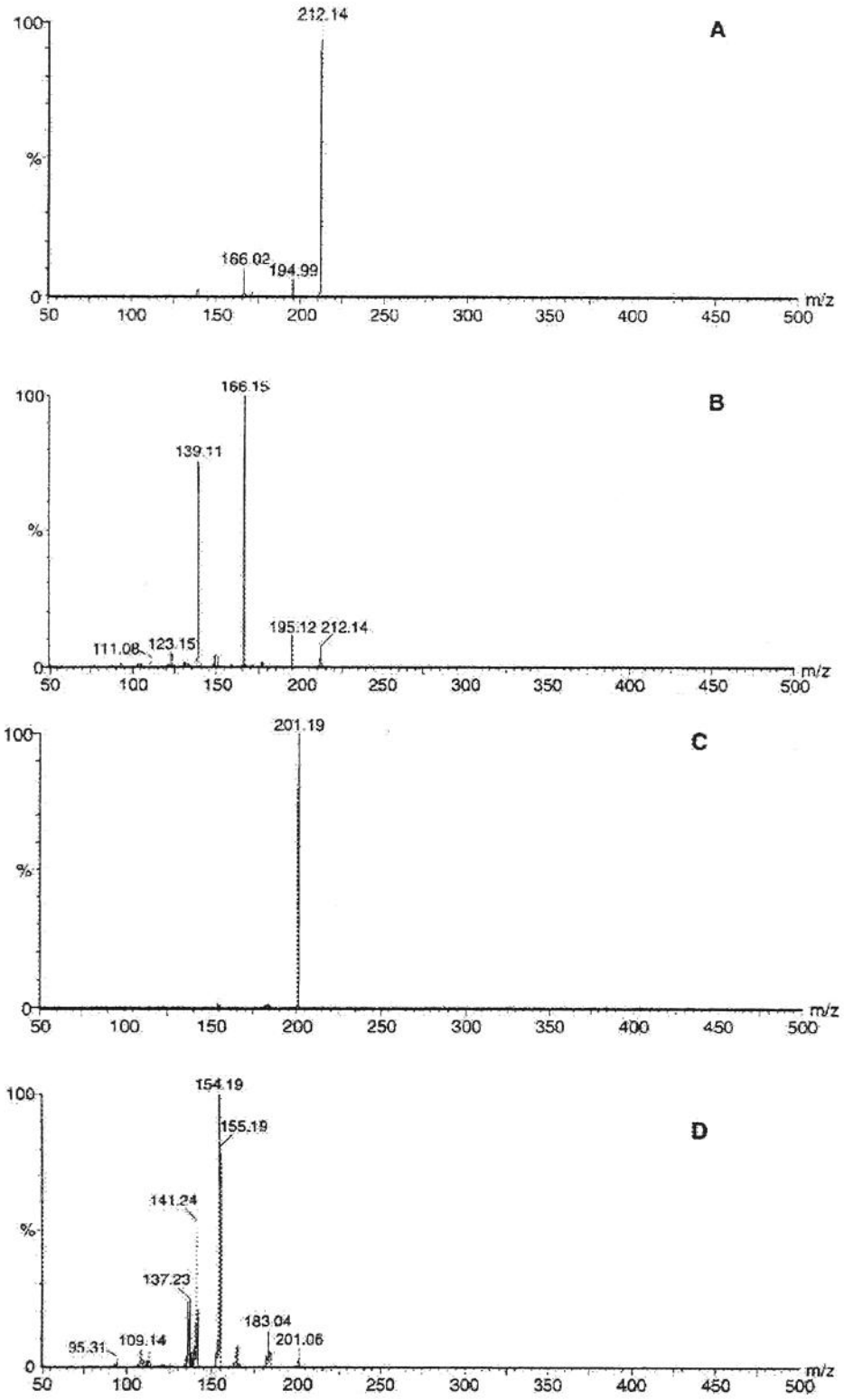
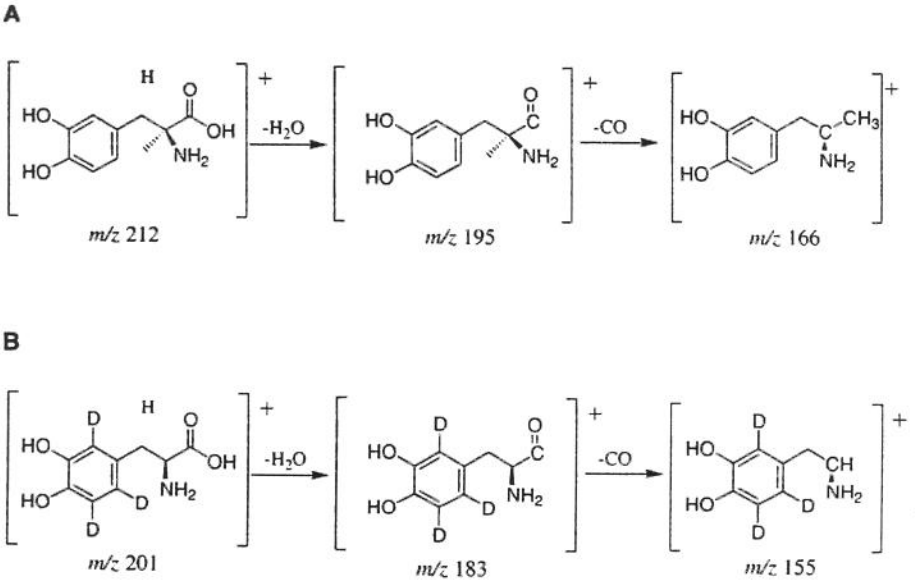


Fig. 1. Full scan mass spectra of (A) methyldopa and (C) I.S. Product ion spectra of (B) methyldopa and (D) I.S.



Scheme 1. Fragmentation route proposed for (A) methyldopa and (B) I.S.

2.6. Stability

Quality control plasma samples (500 and 3000 ng/ml; $n=5$ for each concentration) were subjected to short term (6 h) room temperature, three freeze–thaw (−20 to 25 °C) cycles and 24-h autosampler (5 °C) stability stability tests. Subsequently the methyldopa concentrations were measured comparing with fresh prepared samples and the significance of the obtained results was analyzed by the Student's t-test ($P>0.05$).

2.7. Bioequivalence study

The analytical method was applied to evaluate the bioequivalence of two tablet formulations of methyldopa in healthy volunteers: Legrand Metildopa (test formulation from EMS Indústria Farmacêutica; lot No. 003352, expiration date July 2002) and Aldomet (standard reference formulation from Prodome Química e Farmacêutica; lot No. BB089, expiration date February 2003).

Twenty-five healthy volunteers of both sexes (12 male and 13 female), aged between 18 and 50 years and within 15% of the ideal body mass, were selected for the study after assessment of their health status by clinical evaluation (physical examination, ECG) and the following laboratory tests: blood glucose, urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, γ-gluthamil transferase (γ-GT), total bilirubin, albumin and total protein, triglyceride, total cholesterol, hemoglobin, hematocrit, total and differential white cell counts, and routine urinalysis. All subjects were negative for human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV), except for serological scar.

The volunteers had the following clinical characteristics (divided by gender and expressed as mean±SD [range]): male: age: 34.1±7.0 years [27–45], height: 174.8±8.6 cm [160.5–190.0], body mass: 81.1±9.9 kg [65.0–94.5]; female: age: 30.9±5.2 years [25–39], height: 155.6±5.2 cm [150.0–165.0], body mass: 57.8±7.1 kg [45.5–69.4].

The study was a single dose, two-way randomized crossover design with a 5-day washout period between the doses. During each period, the volunteers were hospitalized at 21:00 h having already had a normal evening meal, and after an overnight fast they received (at 06:00 h) a single dose of methyldopa (500 mg of either tablet formulation). Water (200 ml) was given immediately after the drug administration and the volunteers were then fasted for 4 h, after which period, a standard lunch was served; an evening meal was provided 10 h after dosing. No other food was permitted during the

"in-house" period and liquid consumption was allowed ad libitum after lunch (with the exception of xanthine-containing drinks, including tea, coffee, and cola). Systolic and diastolic arterial pressure (measured non-invasively with a sphygmomanometer), heart rate and temperature were recorded just before and hourly after the administration of each methyldopa dose.

Blood samples (6 ml) from a suitable antecubital vein were collected by indwelling catheter into EDTA containing tubes before and 20, 40, 60, 80, 100 min, 2, 2.5, 3, 4, 5, 6, 8, and 12 h post-dosing. The blood samples were centrifuged at 2000 *g* for 10 min at room temperature and the plasma stored at −20 °C until assayed for methyldopa content.

Bioequivalence between the two formulations was assessed by calculating individual test/reference ratios for the peak of concentration (C_{max}), area under curve (AUC) of plasma concentration until the last concentration observed (AUC_{last}), and the area under curve between the first sample (pre-dosage) and infinite ($AUC_{0-\infty}$). The C_{max} and the time taken to achieve this concentration (T_{max}) were obtained directly from the curves. The areas under the methyldopa plasma concentration vs. time curves from 0 to the last detectable concentration (AUC_{last}) were calculated by applying the linear trapezoid rule. Extrapolation of these areas to infinity ($AUC_{0-\infty}$) was done by adding the value C_{last}/ke to the calculated AUC_{last} (where C_{last} = the last detectable concentration, and ke = the first-order terminal elimination rate constant which was estimated by linear regression from the points describing the elimination phase on a log-linear plot). The AUC and C_{max} data for the two formulations were analyzed by analysis of variance (ANOVA) to establish whether the 90% confidence interval (CI) of the ratios was within the 80–125% interval indicating bioequivalence as proposed by the US Food and Drug Administration.

Parametric and non-parametric analyses of ln-transformed arithmetic means and individual T_{max} differences between test and reference formulations were performed.

3. Results

Under the LC–MS–MS conditions previously described, and as shown in Fig. 2A–C, no endogenous peak was observed in the mass chromatogram of blank plasma. The mass chromatograms of a sample are shown in Fig. 3, in which both the retention times of methyldopa and I.S. were 3.4 min. Mean absolute recoveries were calculated as the ratio of the mean response areas for extracted and unextracted (spiked blank plasma extract) samples at the same concentration expressed as a percentage. The recoveries observed were (means±SD) 93±5, 89±7 and 83±11% (25, 250 and 2500 ng/ml, respectively) for methyldopa, and 80±11% for I.S. (2000 ng/ml).

Stability analysis was performed with plasma

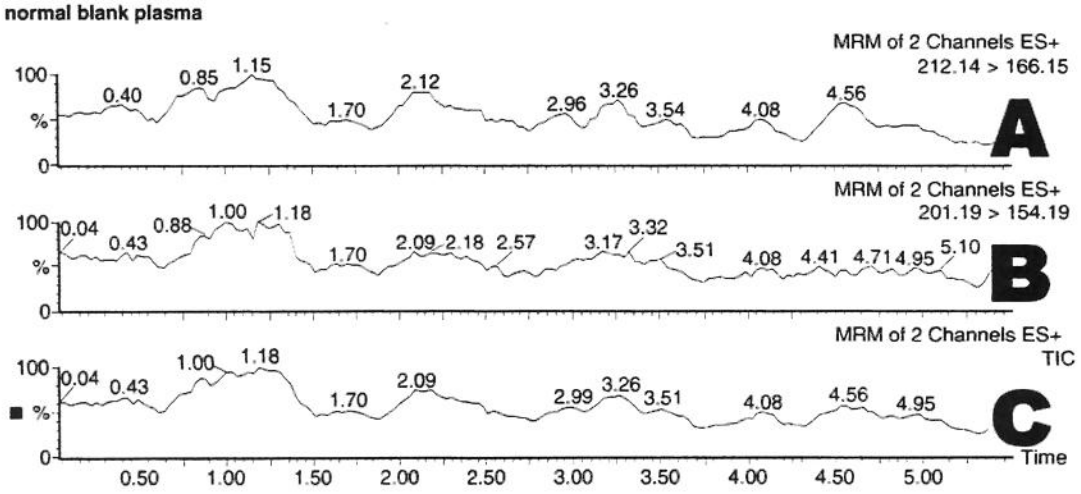


Fig. 2. MRM chromatograms of blank human plasma: (A) methyldopa, (B) I.S., and (C) total ion chromatogram (TIC).

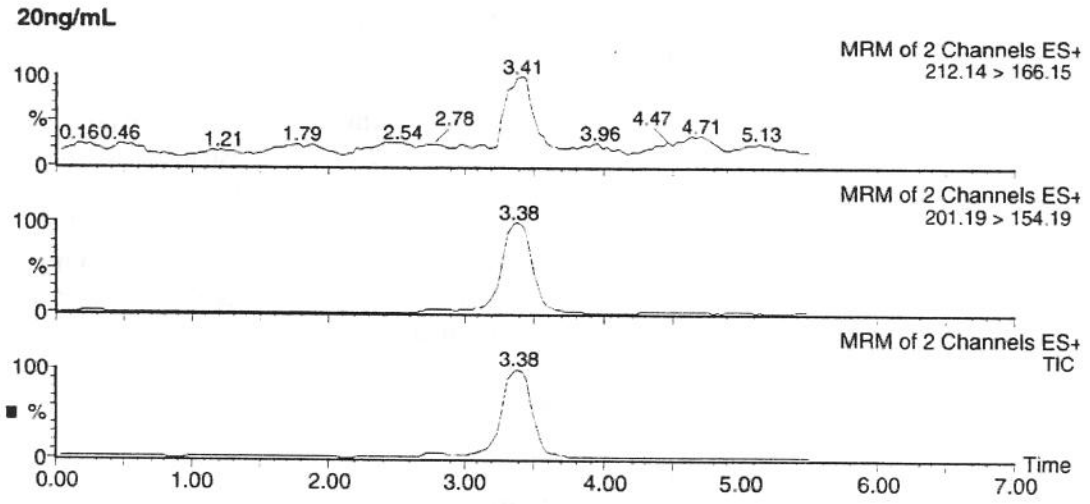


Fig. 3. MRM chromatograms of methyldopa, I.S. and TIC.

quality control samples (500 and 3000 ng/ml). All samples showed no significance degradation under the conditions previously described in the Experimental section.

3.1. Assay performance

A linear least-squares regression with a weighting index of $1/x$ was performed on the peak area ratios of methyldopa and I.S. vs. methyldopa concentrations of the eight human plasma standards (in duplicate) to generate a calibration curve. Calibration curves showed linear relationship between peak area ratios (methyldopa/internal standard) and α-methyl DOPA concentrations ($y=ax+b$, where y=peak area ratio and x=methyldopa concentration in ng/ml). Mean (SD) values for coefficients a and b obtained along the study were 0.00058 (0.00008) and 0.036 (0.007), respectively; regression coefficient (r) values varied from 0.992 and 0.997 (mean: 0.995, SD: 0.001). The calibration curve was linear over the range 20 to 5000 ng/ml. The mean coefficient of correlation (r) was greater than 0.997.

The lower limit of quantification (LOQ), defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, was 20 ng/ml.

The precision and accuracy of quality control samples are shown in Table 1. Precision was determined as the percent relative standard deviation, RSD (%)=100·(SD/M) and accuracy as the percent relative error, RE (%)=$(E-T)\cdot(100/T)$, where M is the mean, SD is the standard deviation, T is theoretical concentration and E is the experimentally determined concentration.

Two volunteers dropped out the study (one female because difficult in getting and keeping a venous access, and one male because right foot cellulitis which required medication). Six volunteers (four female) reported headache that was considered mild and of short duration; one male volunteer needed to take medication (paracetamol). Five volunteers (four female) reported drowsiness that was also considered mild. Additionally, three volunteers (two female) reported mild dizziness and one female volunteer reported mild nausea.

Table 1
Intra- and inter-batch variation of analytical data

	QL1	QCA	QCB	QCC
Intra-batch				
Nominal concentration (ng/ml)	20	30	600	3000
Mean	19	28	592	2941
SD (n=8)	1.6	2.0	31.9	127.9
Accuracy (%)	−7.1	−8.0	−1.3	−2.0
Precision (%)	8.6	7.3	5.4	4.3
Inter-batch				
Nominal concentration (ng/ml)	20	30	600	3000
Mean	20	30	593	2930
SD (n=3)	1.5	2.0	3.2	19.0
Accuracy (%)	1.1	0.2	−1.1	−2.3
Precision (%)	8.9	7.7	0.5	0.7

Table 2
Mean pharmacokinetic parameters obtained from 23 volunteers after administration of 500 mg methyldopa tablet

Parameter	Aldomet	Metildopa
AUC_{last} ([ng h]/ml)		
Geometric mean	6365	6651
SD	2917	3587
$AUC_{0-12\ h}$ ([ng h]/ml)		
Geometric mean	6545	6862
SD	2904	3643
C_{max} (ng/ml)		
Geometric mean	1303	1358
SD	480	621
$T_{1/2}$ (h)		
Median	2.6	2.2
Range	1.0–7.6	0.6–10.6
T_{max} (h)		
Median	3.0	3.0
Range	1.7–6.0	1.7–6.0

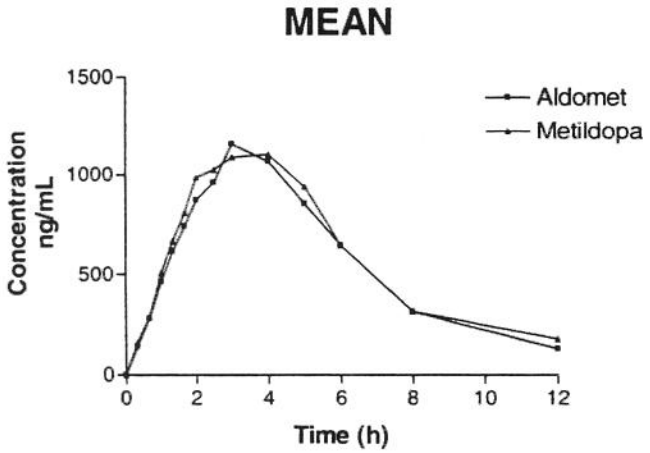


Fig. 4. Mean plasma concentrations vs. time curve for both methyldopa formulations.

Table 2 shows the mean pharmacokinetic parameters obtained after the administration of 500 mg of methyldopa to 23 healthy volunteers. The mean plasma methyldopa concentrations vs. time curves for both preparations are shown in Fig. 4, and the statistical analysis of the bioequivalence parameters and their confidence intervals are recorded in Table 3.

The geometric mean and respective 90% CIs of Legrand Metildopa/Aldomet percent ratios were 98.7% (88.9–103.7%) for C_{max}, 97.0 (85.0–110.7%) for AUC_{last}, and 100.5% (87.9–114.8%) for $AUC_{0-\infty}$ (Table 3). T_{max} was also statistically analyzed and the point estimate for individual differences (Legrand Metildopa/Aldomet) was 0.0 h (90% CI of −0.7 to 0.5).

4. Discussion

Although methyldopa plasma concentrations have been measured through different analytical methods, the use of HPLC seems the more frequently used [2–7]. Róna et al. [3] obtained an LOQ of 10 ng/ml using solid-phase extraction and HPLC–fluorescence detection but the retention time and the total run time were very long (11.4 and 35 min, respectively). Lucarelli et al. [4], using dual working electrode coulometric detection, obtained an LOQ of 1.6 ng/ml, but the total run time was also long (>20 min) and the extraction was more complex. Other electrochemical detection have been reported [5–7] in which the retention times were approximately 10 min and the extraction methods used were more complex than our method. The LOQ observed ranged between 50 to 200 ng/ml.

In the present study, the retention time and the

Table 3
Geometric means of the individual AUC_{last}, $AUC_{0-\infty}$, and C_{max} ratios (test/reference formulation) and the respective 90% confidence intervals (CIs)

Metildopa/Aldomet	Statistical analysis			
	Parametric		Non-parametric	
	Geometric mean	90% CI	Geometric mean	90% CI
AUC_{last} % ratio	97.0	85.0–110.7	99.8	88.7–114.4
$AUC_{0-\infty}$ % ratio	100.5	87.9–114.8	104.4	91.1–118.3
C_{max} % ratio	98.7	88.9–103.7	100.4	88.8–112.5

total run time for methyldopa were shorter (5.5 and 3.3 min, respectively) than those previously reported assays. We decided to use deuterium labeled L-dopa for avoiding possible endogenous interference providing from the plasma, although some authors have reported the use of unlabeled dopa [3]. On the other hand, the extraction procedure in our case is simple and rapid and do not include a concentration step. The LOQ observed in the present study (20 ng/ml) was higher than reported in other studies but it was considered sufficient for the bioequivalence study.

After oral administration of the methyldopa tablets to the volunteers, the observed methyldopa peak plasma concentration (C_{max}) values and the time values taken to be achieved (T_{max}) were similar to those reported in the literature and equivalent between the formulations. In addition, the calculated 90% CIs for mean C_{max}, AUC_{last} and $AUC_{0-\infty}$ Legrand Metildopa/Aldomet individual ratios were within the 80–125% interval defined by the US Food and Drug Administration [8].

5. Conclusion

An LC–MS–MS method for the quantification of methyldopa in human plasma was developed and validated. The method satisfied the requirements of high sensitivity, specificity and rapid sample throughput that are necessary for pharmacokinetic studies.

The pharmacokinetic data demonstrated that the methyldopa test formulation (Legrand Metildopa) is bioequivalent in terms of both rate and extent of absorption to reference formulation (Aldomet).

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Amlodipine Bioequivalence Study: Quantification by Liquid Chromatography Coupled to Tandem Mass Spectrometry

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ABSTRACT: *Objective*—To assess the bioequivalence of two amlodipine tablet formulations (Amlodipine® 5 mg tablet from Merck S.A. Indústrias Químicas, Brazil as test formulation and Norvasc® 5 mg tablet from Laboratórios Pfizer Ltd., Brazil as reference formulation) in 24 healthy volunteers of both sexes.

Methods—The study was conducted using an open, randomized two-period crossover design with a 4-week washout interval. Plasma samples were obtained over a 144 h period. Plasma amlodipine concentrations were analyzed by combined liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) with positive ion electrospray ionization using multiple reaction monitoring (MRM). From the amlodipine plasma concentration vs time curves, the following pharmacokinetic parameters were obtained: AUC_{last}, AUC_{0-inf} and C_{max}. The statistical interval proposed was 80–125% according to the US Food and Drug Administration Agency.

Results—The limit of quantification was 0.1 ng/ml for plasma amlodipine analysis. The geometric mean and the 90% confidence interval (CI) test/reference ratios were 101.2 (92.9–110.2%) for AUC_{last}, 99.6 (91.5–108.4%) for AUC_{0-inf} and 98.5 (89.0–109.1%) for C_{max}.

Conclusion—Since the 90% CI for AUC_{last}, AUC_{0-inf} and C_{max} ratios were within in the 80–125% interval proposed by the US FDA, it was concluded that Amlodipine® 5 mg tablet (test formulation) was bioequivalent to Norvasc® 5 mg tablet, in terms of both rate and extent of absorption.

Key words: pharmacokinetics; desipramine; HPLC; LC-MS-MS

Introduction

Amlodipine, 2-[(2-Aminoethoxy)methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl 5-methyl ester, a third-generation dihydropyridine calcium antagonist, is used to lower blood pressure in hypertensive patients [1]. It has an average molecular weight of 408.9 g/mol, and is commercially available for clinical use as 5 and 10 mg tablets.

The aim of this study was to assess the bioequivalence in healthy human volunteers of both sexes, of two amlodipine 5 mg tablet formulations. Amlodipine and the internal standard desipramine (IS) (Figure 1) plasma levels were measured using a high performance liquid chromatography assay coupled to tandem mass spectrometry (LC-MS-MS).

Methods

Clinical protocol

Twenty-four healthy volunteers of both sexes, aged between 18 and 45 years old and within the

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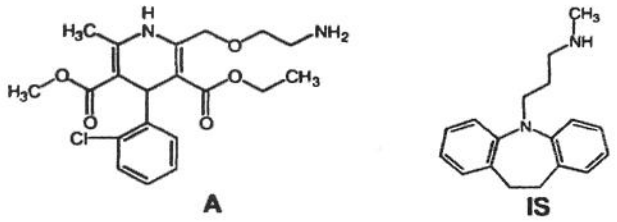

Figure 1. Chemical structure of amlodipine (A) and internal standard desipramine (IS)

15% of the ideal body weight were selected for the study. The volunteers were free from significant cardiac, hepatic, renal, pulmonary, neurological, gastrointestinal and hematological diseases, as assessed by physical examination, ECG, and the following laboratory tests: blood glucose, urea, creatinine, AST, ALT, alkaline phosphatase, γ-GT, total bilirubin, uric acid, total cholesterol, triglycerides, albumin and total protein, hemoglobin, hematocrit, total and differential white cell counts, erythrocyte sedimentation rate and routine urinalysis. All subjects were negative for HIV, HBV (except for serological scar) and HCV.

The male group was composed of 12 volunteers (21.8 $\pm$ 2.8, mean $\pm$ s.d.m; range 18–27 years), height between 160 and 186 cm (172.9 $\pm$ 7.2 cm), weighing between 55.0 and 90.5 kg (73.0 $\pm$ 11.8 kg). The female group was also composed by 12 volunteers (21.8 $\pm$ 5.0 year; range: 20–36 years), height between 158 and 173 cm (164.3 $\pm$ 5.0 cm), weighing between 50.0 and 73.2 kg (59.6 $\pm$ 7.6 kg).

All subjects gave a written informed consent, and the Ceará Federal University Hospital Ethics Committee of Clinical Investigation approved the clinical protocol.

The study was conducted in an open, randomized, two-period crossover fashion with a four-week washout interval between doses. During each period, the volunteers were hospitalized at 10:00 p.m., having already eaten a normal evening meal, and after an overnight fast they received at 7:00 a.m. a single 5 mg dose of the appropriate amlodipine tablet formulation along with 200 ml of tap water. No food was allowed during 3 h following drug administration, after which a standard breakfast was consumed. A lunch and an evening meal were provided 5 and 10 h after dosing, respectively. No other food was permitted during the 'in-house' period. Liquid consumption was permitted *ad libitum* after lunch, but xanthine-containing drinks including tea, coffee, or cola were avoided.

At each blood sampling time, systolic and diastolic arterial pressure (measured non-invasively with a sphygmomanometer) and heart rate were recorded.

Formulations

The following formulations were employed: Amlodipine® from Merck S.A. Indústrias Químicas, Brazil (lot number 99Z047, expiration date 03/2001) as test formulation, and Norvasc® from Laboratórios Pfizer Ltd., Brazil (lot number 804-05034 & 904-05002-B, expiration date 10/2000 & 01/2001, respectively) as reference formulation.

Chemicals and reagents

Amlodipine (hydrochloride form) was provided by Merck Indústria e Comercio Ltda, (Brazil) and desipramine was obtained from Sigma (USA). The following analytical or HPLC grade reagents were used: ammonia solution from Synth (Brazil), diethyl-ether from Jand Química Ind. & Com. Ltd. (Brazil), acetonitrile from Nuclear (Brazil), formic acid, sodium carbonate and sodium bicarbonate from Mallinckrodt (USA), acetic acid glacial from Química Moura (Brazil), hexane from Quimex (Brazil), methyl alcohol from J.T. Baker (Brazil) and water (purified using Milli-Q or Elga UHQ systems). Pools of human plasma for quality control and calibration curve preparations were provided by São Paulo University Hospital, Brazil.

Calibration standards and quality control

Stock solutions of amlodipine and internal standard (desipramine) were prepared in methanol–water (50:50 v/v) at a concentration of 1 mg/ml. Calibrations curves of amlodipine were prepared in blank plasma at concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 ng/ml and performed in duplicate for each batch. The quality control samples were prepared in blank

plasma at concentrations of 0.5, 2.0 and 10.0 ng/ml (QCA, QCB, and QCC, respectively) independent of the calibration standards.

Drug analysis

Blood samples (10 ml) from a suitable antecubital vein were collected into EDTA-containing tubes before and 1, 2, 4, 6, 8, 10, 12, 14, 24, 48, 96, 120, and 144 h after the administration of each dose of amlodipine. The blood samples were centrifuged at 2500 *g* for 10 min at room temperature and the plasma was decanted and stored at −20°C until assayed for their amlodipine contents.

All frozen human plasma were previously thawed at ambient temperature and centrifuged at 2000 *g* for 5 min at 4°C to precipitate solids. Two hundred µl of IS solution (10 ng/ml desipramine in carbonate buffer pH 9.0) were added to 200 µl aliquot of plasma sample. The tubes were briefly vortex-mixed and the compounds of interest were extracted with 4 ml of diethylether/hexane (80:20 v/v). The mixture was vortex-mixed for approximately 40 s, and the organic phase was evaporated under N_2 at 37°C. The dry residues were reconstituted with 200 µl of mobile phase (60% CH_3CN; 40% H_2O; plus 10 mM formic acid) and transferred to the autoinjector microvials.

Chromatographic conditions

An aliquot (40 µl) of each plasma extract was injected into a Genesis C_{18} 4 µm analytical column (150 mm × 4.6 mm i.d.; Jones Chromatography) operated at a temperature of 40°C. The compounds were eluted by pumping the mobile phase at a flow rate of 0.4 ml/min. Under these conditions, typical standard retention times (RT) were 3.4 min for amlodipine and 3.6 min for desipramine, and back-pressure values of approximately 35–45 bar were observed.

A split of the column eluant of approximately 1:10 was included so that only approximately 40 µl/min entered into the mass spectrometer. The temperature of the autosampler was kept at room temperature (22–25°C) and the total run time was 5.0 min.

Mass-spectrometry conditions

The mass spectrometer (Micromass Quattro LC) equipped with an electrospray source using a crossflow counterelectrode was run in positive mode (ES+), and set up in Multiple Reaction Monitoring (MRM), monitoring the transitions 409.0>238. 1 and 267.1>236.2 for amlodipine and IS, respectively. For both amlodipine and IS, the capillary voltage, the dwelling time, the cone voltage and the gas pressure (argon) were 3.5 kV, 0.8 s, 20 V and 7.4×10^{-4} mbar, respectively. The collision energy was 11 eV for amlodipine and 15 eV for desipramine. The percent of precursor ion attenuation, under the mass spectrometer conditions described above, were 99 and 98% for analyte and IS, respectively. Data acquisition and analysis were performed using the software MassLynx (v 3.2 running under Windows NT (v 4.0) on Pentium PC).

Method development

Full-scan positive mass spectra of amlodipine and IS showed the protonated molecules, $[M-H]^+$, of *m/z* 409 and 267, respectively, according to the previous study [2]. The most abundant ion in the product ion spectra was at *m/z* 238 for amlodipine obtained by an unusual fragmentation mechanism elucidated in the same study [2]. The *m/z* 236 was the most abundant product ion for IS as a result of neutral loss of methylamine (CH_3NH_2). The structures proposed for both product ions are illustrated in Figure 2.

From these results, the mass spectrometer was set as follows: *m/z* 409 for amlodipine and *m/z* 267 for desipramine as the precursor ions and *m/z* 238 and 236 as the respective product ions in the MRM mode. No peak was observed in the mass chromatogram of blank human plasma under the LC-MS-MS conditions described above, as shown in Figure 3A. Also, the mass chromatograms of a sample are shown in Figure 3B, where it can be observed that the retention times of amlodipine and IS were 3.4 and 3.5 min, respectively.

Linearity, precision and accuracy were determined to assess the performance of the method. A linear least-squares regression with a weighting index of $1/x$ was performed on the peak area ratios of amlodipine and IS vs amlodipine

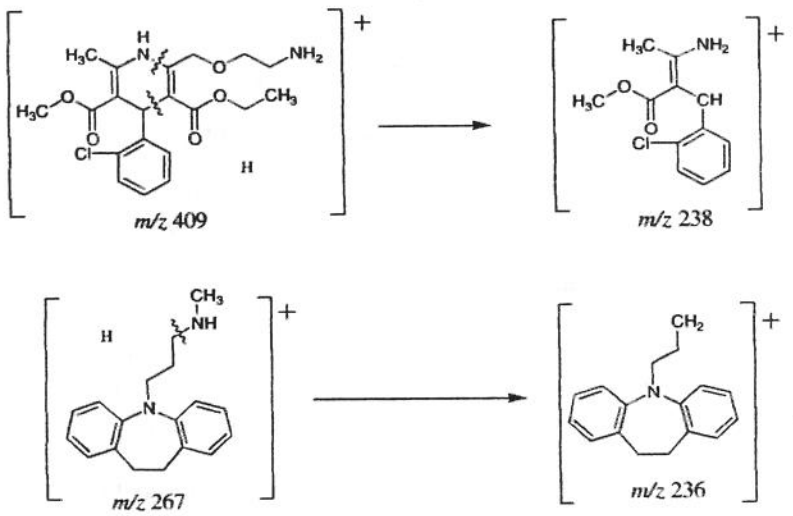

Figure 2. Dissociation route proposed for amlodipine and desipramine

concentrations of the human standards (in duplicate) to generate a calibration curve.

A quality control sample (QCA, QCB or QCC) was analyzed after a sequence of 10 unknown samples. The lowest limit of quantification (LOQ) was defined as the lowest concentration at which both precision and accuracy were less than 20%.

Stability

Quality control samples (1.0 and 10.0 ng/ml) were subject to short-term room temperature (6 h), three freeze/thaw cycles, 24 h-autosampler stability and long-term stability (15 days) tests. Subsequently, the amlodipine concentrations were measured compared with freshly prepared samples. The significance of the obtained results was analyzed by the Student's *t*-test ($p>0.05$).

Pharmacokinetics and statistical analysis

The first-order terminal elimination rate constant (ke) was estimated by linear regression from the points describing the elimination phase on a log-linear plot. Half-life ($t_{1/2}$) was derived from this rate constant [$t_{1/2}=\ln(2)/ke$]. The maximum observed plasma concentration (C_{max}) and the time taken to achieve this concentration (T_{max}) were obtained directly from the curves. The areas under the amlodipine plasma concentration vs time curves from 0 to the last detectable concentration (AUC_{last}) were calculated by applying the linear trapezoid rule. Extrapolation of these areas to infinity (AUC_{0-inf}) was done by adding the value C_{last}/ke to the calculated AUC_{last} (where C_{last} = the last detectable concentration).

For T_{max} statistical analysis, both the arithmetic mean and the individual T_{max} differences between test and reference formulations were used. Parametric and non-parametric analysis on ln-transformed data were done.

Results and Discussion

Tolerance of both formulations was good. Six volunteers complained of headache and one volunteer of dysmenorrhea. All biochemical parameters monitored presented no clinically relevant alterations.

The calibration curve showed good linearity within 0.1–20 ng/ml of amlodipine ($r^2>0.998$) (Figure 4). The limit of quantification was 0.1 ng/ml for amlodipine. The intra-batch precision were 5.7, 4.4, and 5.6% for 0.5, 2.0 and 10.0 ng/ml, respectively. The intra-batch accuracy were −1.0, −4.2, and 1.3%, respectively. The

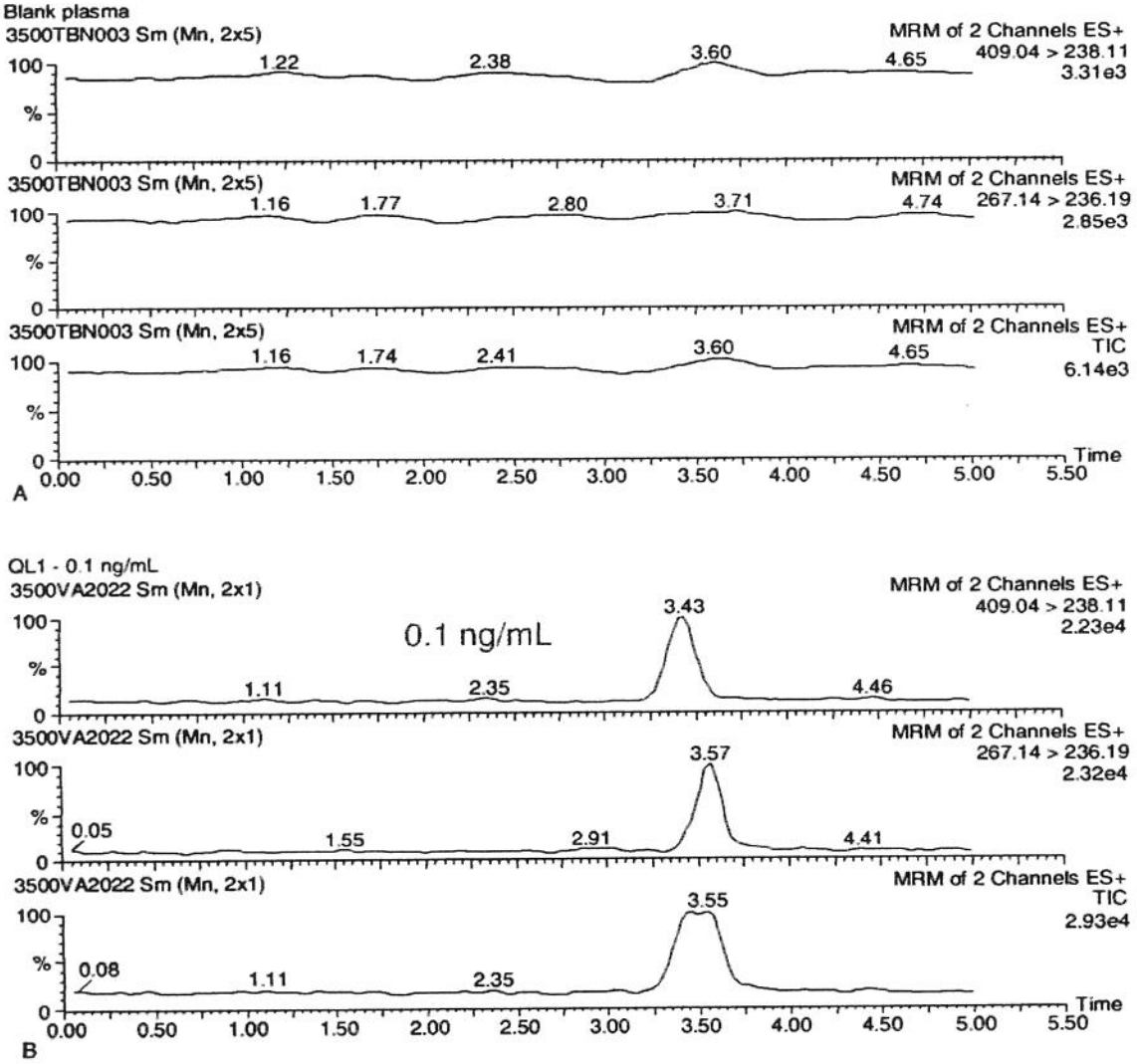

Figure 3. Selected ion chromatograms (MRM) of 2 channels ES+. A: blank human plasma for amlodipine, desipramine and total ion count (TIC). B: 409.0>238.1 for amlodipine and 267.1>236.2 for desipramine. The peaks illustrate the retention time and integrated area

inter-batch precision for the same concentrations were 0.9, 5.1, and 1.0%, respectively, and the inter-batch accuracy were 0.0, −1.5, and 0.2%, respectively (Table 1).

The recovery of amlodipine, based on peak areas are ratios of extracted normal human plasma/mobile phase, both previously spiked at final concentrations of 1.0 and 10.0 ng/ml, were 63.7 and 77.9% ($n = 5$), respectively. For the IS (10 ng/ml), the recovery was 52.7% ($n = 10$). No plasma matrix effect was observed under the described extraction procedure.

Stability analysis was performed with quality control samples (1.0 and 10.0 ng/ml). All samples showed no significant degradation.

The mean amlodipine plasma concentrations vs time profiles after a single oral dose of each 5 mg tablet formulation of amlodipine are shown in Figure 5. Table 2 shows the mean pharmacokinetic parameters obtained from 24 volunteers after the administration of 5 mg amlodipine tablet. Table 3 presents the ratios and the respective 90% confidence intervals for bioequivalence analysis. The geometric mean and

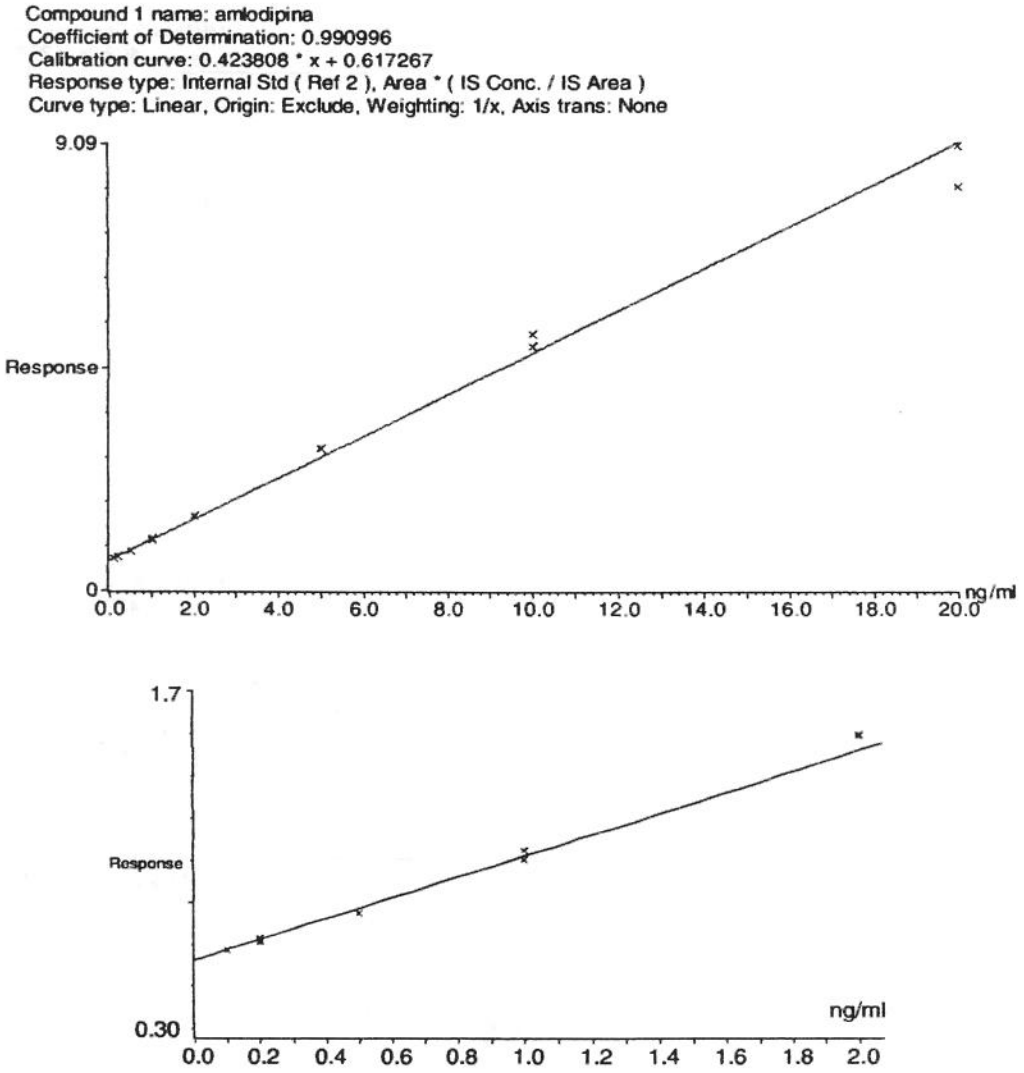

Figure 4. Calibration curve of amlodipine

respective 90% confidence interval (CI) of Amlodipine®/Norvasc® percent ratios were 98.5% (89.0–109.1%) for C_{max}, 101.2 (92.9–110.2%) for AUC_{last}, and 99.6% (91.5–108.4%) for AUC_{0-inf}.

T_{max} was also statistically analyzed and the point estimate for individual differences (Amlodipine® vs Norvasc®) was 0.0 h (90% CI of −1.0 to 2.0) (Table 3).

Amlodipine plasma and serum levels have been detected by different methods such as thin-layer chromatography (LOQ = 2.0 ng/ml) [1], liquid chromatography with amperometric detection (LOQ = 0.2 ng/ml; RT = 8.5 min) [3], and liquid chromatography with ultraviolet detection (LOQ = 0.2 ng/ml; RT > 40 min) [4]. Other methods such as capillary electrophoresis (LOQ = 5.0 ng/ml; RT = 9.8 min) [5], and gas chromatography (LOQ = 2.5 ng/ml; RT > 9.0 min) [6] were also used to detect amlodipine plasma levels.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) was recently used to quantify plasma and serum amlodipine

Table 1. Quantified concentration (ng/ml) of individual samples of intra-batch and inter-batch validation

Sample	LOQ	QL2	QCA	QCB	QCC
Intra-batch					
Nominal concentration (ng/ml)	0.1	0.2	0.5	2.0	10.0
Mean	0.1	0.2	0.5	1.9	10.1
S.D.	0.00	0.02	0.03	0.08	0.57
Precision (%)	4.1	7.4	5.7	4.4	5.6
Accuracy (%)	2.3	1.7	1.0	4.2	1.3
Inter-batch					
Nominal concentration (ng/ml)	0.1	0.2	0.5	2.0	10.0
Mean	0.1	0.2	0.5	2.0	10.0
S.D.	0.00	0.01	0.00	0.12	0.10
Precision (%)	1.2	4.4	0.9	5.1	1.0
Accuracy (%)	0.9	2.4	0.0	−1.5	0.2

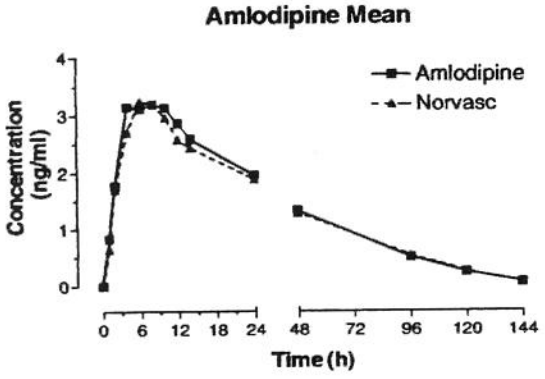

Figure 5. Mean plasma concentrations vs time curve for both amlodipine formulations

concentration [2,7]. These methodologies improved the specificity, accuracy and in shortening the run time. Yasuda *et al.* [2] using an LC-MS-MS with atmospheric pressure chemical ionization (APCI) obtained an LOQ of 0.014 ng/ml. The molecular fragmentation of amlodipine was the same as that in our study (*m/z* 409.0>238.1). Besides the low LOQ, the retention time was longer (4.5 min) than our method and used a deuterated internal standard which is seldom commercially available. Marzo *et al.* [7], developed an LC-MS-MS method using nitrendipine as internal standard. The LOQ was the same (0.1 ng/ml) and the retention time was shorter (2.0 min). However, in the sample extraction step they use higher volume of plasma (1.0 ml) than in our study (200 µl).

Table 2. Mean pharmacokinetic parameters obtained from 24 volunteers after administration of 5 mg amlodipine tablet

Pharmacokinetic parameter	Amlodipine®	Norvasc®
$AUC_{(0-last)}$ (ng h/ml)		
Geom. mean	151.7	147.4
S.D.	78.1	75.1
$AUC_{(0-inf)}$ (ng h/ml)		
Geom. mean	166.9	166.3
S.D.	78.8	76.7
C_{max} (ng/ml)		
Geom. mean	3.9	3.8
S.D.	2.5	2.1
Ke (1/h)		
Median	0.02	0.02
Range	0.01–0.03	0.01–0.04
$T_{1/2}$ (h)		
Median	33.9	37.0
Range	24.3–45.7	18.9–63.4
T_{max} (h)		
Median	6.0	6.0
Range	2.0–14.0	4.0–14.0

The method reported here had a good sensitivity and was used to analyze two amlodipine tablet formulations in a bioequivalence study in healthy volunteers using human plasma for the determination of amlodipine concentrations.

After the oral administration of the amlodipine tablets to the volunteers, the observed amlodipine peak plasma concentration (C_{max}) values

Table 3. Geometric mean of the individual AUC_{last}, AUC_{0-inf}, (test/reference formulation) and the respective 90% CI

Amlodipine® /Norvasc®	Parametric		Non-parametric	
	Geom. mean	90% CI	Geom. mean	90% CI
$AUC_{(0-last)}$% ratio	101.2	92.9–110.2	100.6	92.4–109.7
$AUC_{(0-inf)}$ % ratio	99.6	91.5–108.4	104.2	95.8–113.4
C_{max} % ratio	98.5	89.0–109.1	98.8	89.2–109.4
T_{max} (h) individual differences	−0.2 (Arith. mean)	−1.5–1.2	0.0 (Point estimate)	−1.0–2.0

and the time values taken to be achieved (T_{max}) were similar to those reported [2,8] and equivalent between the formulations. In addition, the calculated 90% CI for mean C_{max}, AUC_{last} and AUC_{0-inf} Amlodipine® /Norvasc® individual ratios were within the 80–125% interval defined by the US Food and Drug Administration [9,10]. Thus, it is concluded that amlodipine test formulation (Amlodipine®) is bioequivalent in terms of both rate and extent of absorption to reference formulation (Norvasc ®).

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Nevirapine quantification in human plasma by high-performance liquid chromatography coupled to electrospray tandem mass spectrometry. Application to bioequivalence study

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A rapid, sensitive and specific method to quantify nevirapine in human plasma using dibenzepine as the internal standard (IS) was developed and validated. The method employed a liquid–liquid extraction. The analyte and the IS were chromatographed on a C_{18} analytical column, (150 × 4.6 mm i.d. 4 μm) and analyzed by tandem mass spectrometry in the multiple reaction monitoring mode. The method had a chromatographic run time of 5.0 min and a linear calibration curve over the range 10–5000 ng ml^{-1} (r^2 > 0.9970). The between-run precision, based on the relative standard deviation for replicate quality controls was 1.3% (30 ng ml^{-1}), 2.8% (300 ng ml^{-1}) and 3.6% (3000 ng ml^{-1}). The between-run accuracy was 4.0, 7.0 and 6.2% for the above-mentioned concentrations, respectively. This method was employed in a bioequivalence study of two nevirapine tablet formulations (Nevirapina from Far-Manguinhos, Brazil, as a test formulation, and Viramune from Boehringer Ingelheim do Brasil Química e Farmacêutica, as a reference formulation) in 25 healthy volunteers of both sexes who received a single 200 mg dose of each formulation. The study was conducted using an open, randomized, two-period crossover design with a 3 week washout interval. The 90% confidence interval (CI) of the individual ratio geometric mean for Nevirapina/Viramune was 96.4–104.5% for $AUC_{(0\text{-last})}$, 91.4–105.1% for $AUC_{(0\text{-}\infty)}$ and 95.3–111.6% for C_{max} (AUC = area under the curve; C_{max} = peak plasma concentration). Since both 90% CI for $AUC_{(0\text{-last})}$ and $AUC_{(0\text{-}\infty)}$ and C_{max} were included in the 80–125% interval proposed by the US Food and Drug Administration, Nevirapina was considered bioequivalent to Viramune according to both the rate and extent of absorption.

KEYWORDS: liquid chromatography/tandem mass spectrometry; high-performance liquid chromatography; dibenzepine; bioanalysis

INTRODUCTION

Nevirapine, 11-cyclopropyl-5, 11-dihydro-4-methyl-6*H*-dipyrido [3,2-*b*:2′,3′-*e*][1,4]diazepin-6-one (Fig. 1), is a non-nucleoside reverse transcriptase (RT) inhibitor for the treatment of HIV-1 infected patients developed by Boehringer Ingelheim Pharmaceuticals. It binds directly to the RT catalytic site blocking the RNA- and DNA-dependent DNA polymerase activity.[1–3] Nevirapine (NVP) is a white to off-white crystalline powder with a molecular mass of 266.30 Da and the molecular formula $C_{15}H_{14}N_4O$. It is commercially available in tablets (200 mg) and oral suspensions.

Nevirapine has presented important human immunodeficiency virus antiviral activity at a therapeutic dose of 200 mg twice per day when used in combination with other HIV antiretroviral drugs as protease inhibitors and nucleosides.[4,5] Although nevirapine selects for resistant virus much more rapidly than the current nucleoside analogues,[6] high doses of NVP monotherapy have achieved sustained antiviral activity suppressing immune complex-dissociated HIV p24 antigen and serum HIV RNA.[5]

In clinical studies, NVP has demonstrated excellent bioavailability (>90%) following oral administration of doses up to 400 mg in healthy volunteers and in HIV patients.[7,8] For both area under the curve (AUC) and peak plasma concentration (C_{max}) clinical assays linearity with respect to dose was observed at doses up to 200 mg.[7]

Several methods have been reported for the determination of nevirapine in human plasma or serum. Most of the assays reported for nevirapine determination are based upon high-performance liquid chromatography (HPLC) coupled

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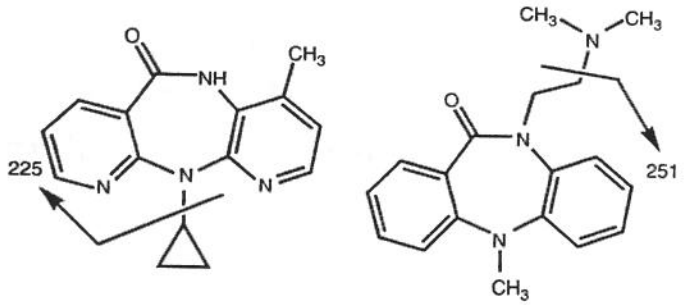

Nevirapine MH^+ 267

Mol. Wt.: 266.30

$C_{15}H_{14}N_4O$

Internal Standard (IS) MH^+ 296

Mol. Wt.: 295.38

$C_{18}H_{21}N_3O$

Figure 1. Proposed mass fragmentation pathways for nevirapine and dibenzepine (IS).

to UV detection, although a single liquid chromatography coupled to mass spectrometry (LC/MS) assay using ion-trap technology has also been reported.[9]

The objective of this study was to develop a specific, sensitive and fast LC/MS/MS method for quantifying nevirapine in human plasma using dibenzepine (Fig. 1) as the internal standard (IS). The method was employed in a bioequivalence study of two different nevirapine oral formulations, Nevirapina (200 mg tablet) provided by Far-Manguinhos, Brazil, as a test formulation, and Viramune (200 mg tablet) produced by Boehringer Ingelheim do Brasil Química e Farmacêutica, as a reference formulation.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile and methanol (HPLC grade) were purchased from Mallinckrodt (Paris, KY, USA) and formic acid (analytical grade) from Merck (Rio de Janeiro, Brazil). Ultrapure water was obtained from an Elga UHQ system (Elga, Bucks, UK). Nevirapine was provided by Far-Manguinhos as a secondary standard grade and with purity of 99.8%. Dibenzepine hydrochloride was purchased from Biomol Research (USA). Blank human blood was collected with EDTA from healthy, drug-free volunteers. After centrifugation of the blank blood, blank plasma was collected, pooled and stored at ~−20 °C until used.

Calibration standards and quality control samples

Nevirapine standard solutions were prepared by dilution of a stock solution (1000 μg ml^{-1}) with methanol–water (50:50, v/v) to give concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20, 50 and 100 μg ml^{-1}. Dibenzepine standard solution was prepared by dilution of a stock solution (1000 μg ml^{-1}) with methanol–water (50:50, v/v) to give a final concentration, base equivalent, of 0.1 μg ml^{-1}. Calibration standard solutions were prepared by spiking control human plasma with standard solutions containing nevirapine to give concentrations of 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng ml^{-1}. A fixed volume (50 μl) of a single dibenzepine concentration (0.1 μg ml^{-1}) was added to all samples (200 μl of plasma) as the internal standard, thus having a final concentration in the sample of 20 ng ml^{-1}. The calibration standards and blanks were freshly prepared (in duplicate) for each assay and were extracted along with plasma samples and quality controls. Quality control (QC) samples for analytical runs were prepared by spiking control human plasma with 30, 300 and 3000 ng ml^{-1} of nevirapine. One QC sample was thawed for each of the three concentrations in every assay and was extracted along with the plasma samples. There were different QC samples (100, 1000 and 5000 ng ml^{-1}) to test assay recovery and stability.

Sample preparation

Frozen human plasma samples were thawed at ambient temperature and centrifuged at 4550 *g* for 5 min at 4 °C to precipitate solids. A 200 μl volume of plasma was dispensed into glass test-tubes (non-siliconized) with 50 μl of the internal standard solution (0.1 μg ml^{-1} dibenzepine). Samples were vortex mixed for ~10 s. A mixture of diethyl ether–hexane (80:20, v/v, 4 ml) was then added and the samples were vortex mixed for 40 s. The organic layer was removed and then evaporated using a flow of nitrogen at 37 °C. The residue was dissolved with 0.2 ml of mobile phase (Acetonitrile–water, 70:30, v/v, containing 10 mM formic acid) and vortex mixed for 15 s to reconstitute the residues. The solution was transferred into vials using automatic pipettes with disposable plastic tips, capped and placed in an HP 1100 autosampler rack.

Liquid chromatographic and mass spectrometric conditions

An HPLC system (Hewlett-Packard, Avondate, PA, USA, Model 1100) consisting of a binary pump (G1312A), solvent degasser (G1322A), autosampler thermostat (G1330A), autosampler (G1329A) and oven (G1316A) was used for all analyses. The chromatographic system consisted of a Genesis C_{18} analytical column (150 × 4.6 mm i.d., 4 μm) from Jones

Chromatography, (Mid-Glamorgan, Wales, UK) and mobile phase (acetonitrile–water, 70:30, containing 10 mM formic acid). An isocratic solution (at a flow-rate of 0.7 ml min^{-1}) was used to elute the analyte and the internal standard (total run time 5.0 min). The pressure of the system was ~50–70 bar. The eluate was split (1:10) and monitored by MS/MS. The column was operated of 40 °C. The temperature of the autosampler was maintained at 5 °C and the injection volume was 20 µl.

Mass spectrometry was performed using a Quattro II triple-stage quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization (ESI) source operating in the positive ion mode using a crossflow counter electrode and set for the multiple reaction monitoring (MRM) mode. The mass spectrometer was previously calibrated with sodium iodide–cesium iodide solution in the range from m/z 20 to 1000 according to the instrument specifications.

The dwell time was set at 0.5 s, the cone energy was 25.0 V and the collision energy was 23.0 and 15.0 eV for nevirapine and IS, respectively.

Method development

Full-scan positive mass spectra of nevirapine and IS showed the protonated molecules, $[M + H]^+$, of m/z 267 and 296, respectively. The most abundant ion in the product ion spectra was at m/z 225 for nevirapine obtained by neutral loss of the cyclopropane ring moiety (42 Da). The m/z 251 ion was the most abundant product ion for IS as result of neutral loss of 45 Da (dimethylamine). Full-scan mass and product ion spectrograms are illustrated in Fig. 2. The proposed fragmentation pathways are illustrated in Fig. 1.

From these results, the mass spectrometer was set as follows: m/z 267 for nevirapine and m/z 296 for IS as the precursor ions and m/z 225 and 251 as the respective product ion. No peak was observed in the mass chromatogram of blank human plasma under the LC/MS/MS conditions described previously, as shown in Fig. 3. The MRM chromatograms of a sample are also shown in Fig. 3, where it can be observed that the retention times of nevirapine and IS were 3.34 and 2.66 min, respectively.

Bioequivalence study

The method presented here was applied to evaluate, on healthy human volunteers of both sexes, the bioequivalence of two nevirapine 200 mg tablet formulations: Nevirapina from Far-Manguinhos, Brazil (lot number 0007614, expiry date 07/2002), as test formulation, and Viramune from Boehringer Ingelheim Pharmaceuticals (lot number 992477B, expiry date 10/2002), as reference formulation. This comparison was made through the quantification of NVP in plasma. The bioequivalence between the two formulations was assessed by calculating individual peak plasma concentrations (C_{max}) and area under the curve ($AUC_{(0-last)}$ and $AUC_{(0-\infty)}$) ratios (test/reference) together with their mean and 90% confidence intervals (CI) after logarithmic transformation of the data. The inclusion of the 90% CI for the ratio in the 80–125% range (US Food and Drug Administration (FDA)) was analyzed by a parametric (analysis of variance (ANOVA)) method.

Twenty-five healthy volunteers of both sexes aged between 22 and 46 years and within 15% of the ideal body weight were selected for the study. The male group was composed of 13 volunteers (32.8 ± 6.3 years, mean ± SD, range 24–41 years), height between 161 and 179 cm (170.2 ± 5.9 cm), weighing between 55.8 and 104.6 kg (73.7 ± 14.4 kg). The female group was composed of 12 volunteers (30.8 ± 7.4 years; range 22–46 years), height between 152 and 164 cm (159.3 ± 3.7 cm), weighing between 50.3 and 75.2 kg (60.1 ± 7.5 kg).

All subjects gave written informed consent and the Institute of Biomedical Sciences of the University of São Paulo ethics committee approved the clinical protocol. All volunteers were healthy as assessed by general physical examination, ECG and the following laboratory tests: blood glucose, urea, uric acid, creatinine, AST, ALT, alkaline phosphatase, γ-GT, total bilirubin, albumin and total protein, tryglicerides, total cholesterol, hemoglobin, hematocrit, ESR, platelet count, total and differential white cell counts, feces parasitological examination and routine urinalysis. All subjects were negative for HIV, HBV (except for serological scar) and HCV. All female volunteers were negative for pregnancy test (β-HCG).

The study was conducted in an open randomized two-period crossover balanced design with a 3 week washout period between the doses. During each period, the volunteers were hospitalized at 9:00 p.m. having already had a normal evening meal, and after an overnight fast starting from 11:00 p.m. they received at 7:30 a.m. a single 200 mg nevirapine tablet of either formulation. Water (150 ml) was given immediately after drug administration. All volunteers were then fasted for 2 h following the drug administration, after which a standard breakfast was consumed. A standard lunch and an evening meal were provided ~5 and ~10 h, respectively, after dosing. No other food was permitted during the 'in-house' period. Liquid consumption was permitted *ad libitum* after lunch but xanthine-containing drinks including tea, coffee and cola were avoided. All subjects were requested to stay in the clinical unit for a 24 h period after drug administration.

Systolic and diastolic arterial pressure (measured non-invasively with a sphygmomanometer), heart rate and temperature were recorded just before and hourly after drug administration.

Blood samples (6 ml) from a suitable antecubital vein were collected in EDTA-containing tubes before and 1, 2, 3, 3.5, 4, 4.5, 5, 6, 8, 10, 12, 24, 48, 72, 96 and 120 h after the administration of each NVP tablet formulation (200 mg). The blood samples were centrifuged at 2000 *g* for 10 min at room temperature and the plasma was decanted and stored at −20 °C until assayed for their NVP content.

Recovery

Preliminary experiments were conducted to evaluate the recovery with the extraction method described above. The percentage recovery was calculated as the ratio of the peak area for extracted blank plasma spiked at each standard

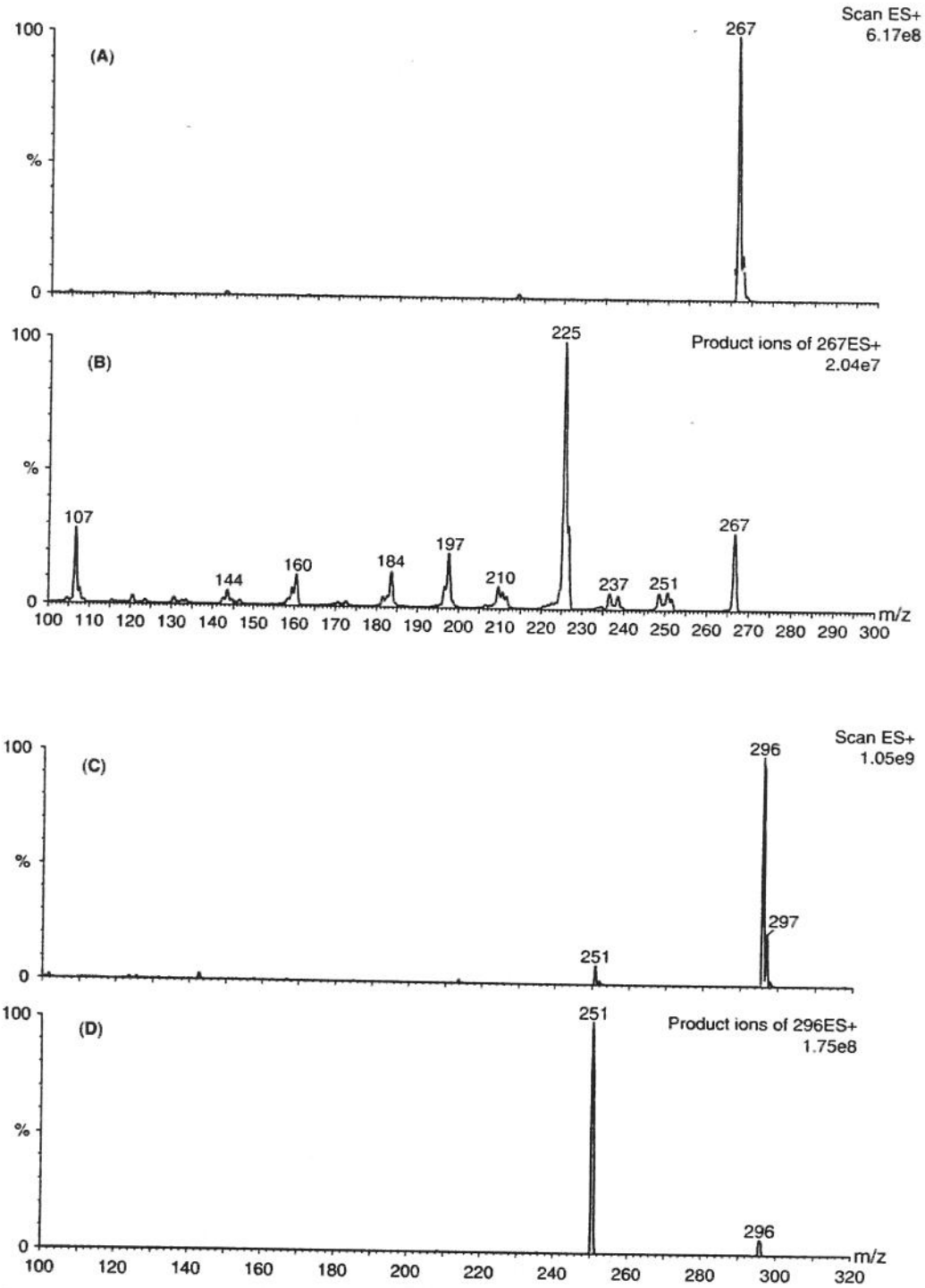

Figure 2. Full-scan mass spectra (CID) of nevirapine (A), dibenzepine (C) and their respective product ion spectra (B and D).

concentration (100, 1000 and 5000 ng ml^{-1}) relative to the peak area of the equivalent mobile phase solutions.

Stability

Quality control samples prepared to test stability (100, 1000 and 5000 ng ml^{-1}) were subjected to short-term (6 h) room temperature, three freeze–thaw cycles and 24 h autosampler (5 °C) stability tests. Subsequently the NVP concentrations were measured in comparison with freshly prepared samples and the significance of the results obtained was analyzed by Student's *t*-test ($p > 0.05$).

Precision and accuracy

The within- and between-run precision were determined as the relative standard deviation, RSD (%) = 100(SD/*M*) and

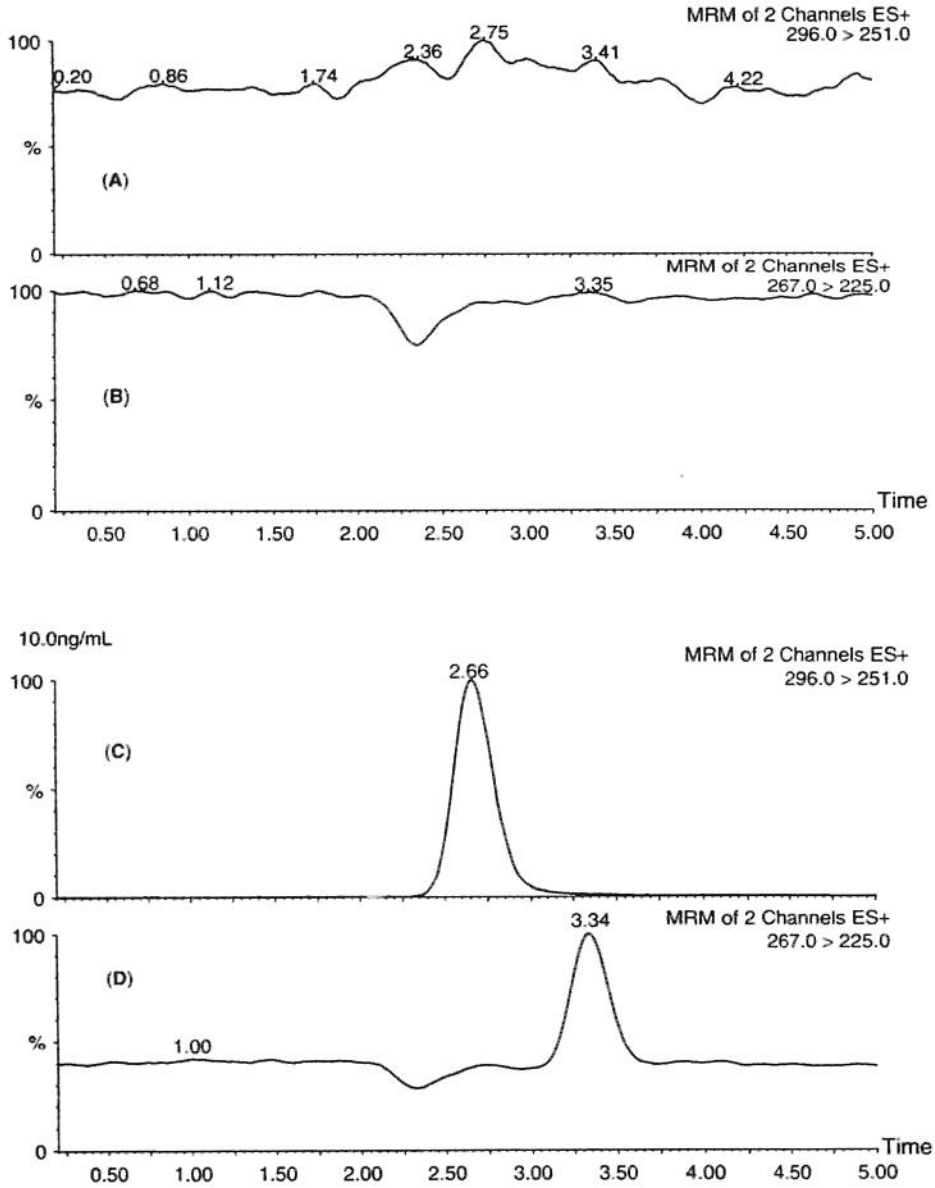

Figure 3. MRM chromatograms of blank pooled human plasma (A and B) and MRM chromatograms of spiked human plasma at a final concentration of 10.0 ng ml^{-1} of dibenzepine (C) and nevirapine (D).

the accuracy as the percentage relative error, RE (%) = $(E - T)(100/T)$, where M is the mean, SD is the standard deviation of M, E is the experimentally determined concentration and T is the theoretical concentration.

Pharmacokinetic and statistical analysis

Plasma nevirapine concentrations were plotted as a function of time and the following pharmacokinetic parameters were obtained for each formulation. The areas under the NVP plasma concentration vs time curves from 0 to last concentration determined ($AUC_{(0\text{-last})}$) were calculated by applying the log-linear trapezoid rule. $AUC_{(0\text{-}120\ h)}$ was calculated by applying the linear trapezoid rule. Extrapolation of these areas to infinity ($AUC_{(0\text{-}\infty)}$) was done by adding the value $C_{120\ h}/K_e$ to the calculated $AUC_{(0\text{-}120\ h)}$ (where $C_{120\ h}$ = plasma concentration calculated from the log-linear regression equation obtained for the estimation of K_e 120 h after dose). The maximum observed plasma concentration (C_{max}) and the time taken to achieve this concentration (T_{max}) were obtained directly from the curves. From the terminal log-decay phase, a first-order elimination rate constant (K_e) was estimated by linear regression and the terminal half-life ($T_{1/2}$) was estimated using the equation $T_{1/2} = \ln(2)K_e^{-1}$.

The 90% CI of the geometric mean for the individual test/reference ratios (Nevirapina/Viramune) for $AUC_{(0\text{-last})}$, $AUC_{(0\text{-}\infty)}$ and C_{max} were obtained to assess the bioequivalence between formulations. Nevirapine was analyzed by using a parametric test (ANOVA for log-transformed data).

For T_{max} statistical analysis, the arithmetic mean and the individual T_{max} differences between test and reference formulations were used. Parametric and non-parametric analyses on log-transformed data were performed.

The software used included WinNonlin Professional Network Edition, version 1.5 m, Bioequivalence Program for Two-Period Crossover Studies, version 3.4, Microsoft Excel version 7, GraphPad Prism version 2.01 and WinSTAT version 3.1.

RESULTS

Assay performance

Linearity, precision and accuracy were determined to assess the performance of the method. A linear least-squares regression with a weighting index of $1/x$ was performed on the peak area ratios of nevirapine/IS vs nevirapine concentrations of the nine human plasma standards (in duplicate) to generate a calibration curve. The method was linear for nevirapine from 10 to 5000 ng ml^{-1} ($r^2 > 0.9970$) on repeated calibration curves.

The recovery of nevirapine, calculated from the peak area ratios of extracted normal human plasma and mobile phase, both previously spiked at final concentrations of 100, 1000 and 5000 ng ml^{-1}, was 106.0 ± 4.2, 100.7 ± 7.5 and 98.6 ± 1.8% (mean ± SD, $n = 5$), respectively. For the IS (20 ng ml^{-1}) the recovery was 101.8 ± 1.2% (mean ± SD, $n = 5$). No matrix effect was observed.

Between- and within-run accuracy and precision for the quality controls are summarized in Table 1. The limit of quantification (LOQ) validated was 10 ng ml^{-1}. The mean nevirapine plasma concentration vs time curves obtained after a single oral dose of each nevirapine tablet formulation are shown in Fig. 4.

Bioequivalence study

Both nevirapine formulations were well tolerated at the administered dose. One male volunteer dropped out the study for personal reasons. The following mild symptoms were reported: one volunteer had headache 7 h after the second 200 mg nevirapine dose (Nevirapina) and one

Table 1. Plasma QC samples generated during routine analysis

		Nominal concentration (ng ml^{-1})		
	Parameter	30	300	3000
Intra-batch	Mean found (ng ml^{-1})	30.8	323	3318
	SD ($n = 8$) (ng ml^{-1})	3.1	17.8	70.2
	Accuracy (%)	2.7	7.7	10.6
	Precision (%)	10.1	5.5	2.1
Inter-batch	Mean found (ng ml^{-1})	31.2	321	3187
	SD ($n = 3$) (ng ml^{-1})	0.4	9.1	116.0
	Accuracy (%)	4.0	7.0	6.2
	Precision (%)	1.3	2.8	3.6

Table 2. Mean pharmacokinetic parameters for 24 volunteers after the administration of nevirapine formulations

Parameter	Nevirapina	Viramune
$AUC_{(0\text{-last})}$ (μg h ml^{-1}):		
Geometric mean	129.6	129.0
90% CI	120.9–138.1	119.3–138.8
$AUC_{(0\text{-}\infty)}$ (μg h ml^{-1}):		
Geometric mean	168.0	171.4
90% CI	150.3–185.7	155.7–187.2
C_{max} (μg l^{-1}):		
Geometric mean	2.1	2.1
90% CI	2.0–2.3	1.9–2.2
K_e (h^{-1})		
Median	0.014	0.01
Range	0.012–0.016	0.008–0.012
$T_{1/2}$ (h):		
Median	50.5	54.2
Range	43.4–57.7	47.0–61.4
T_{max} (h):		
Median	4.4	4.0
Range	2.0–6.8	2.4–5.7

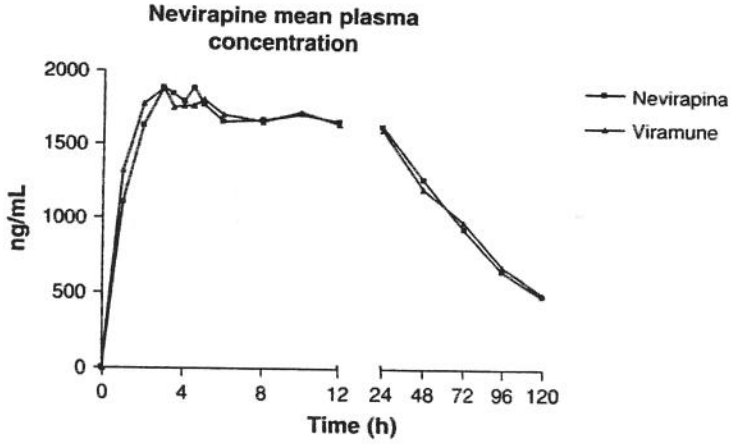

Figure 4. Nevirapine plasma mean concentration versus time profile obtained after single oral administration of 200 mg of the nevirapine tablet formulations.

Table 3. Statistical analysis of individual $AUC_{(0-last)}$, $AUC_{(0-\infty)}$, C_{max} and T_{max} individual difference ratios for both nevirapine formulations

	Statistical analysis			
	Parametric		Non-parametric	
Nevirapina/Viramune	Geometric mean	90% CI	Geometric mean	90% CI
$AUC_{(0-last)}$ % ratio	100.4	96.4–104.5	100.9	96.7–104.6
$AUC_{(0-\infty)}$ % ratio	98.0	91.4–105.1	98.3	91.0–105.6
C_{max} % ratio	103.1	95.3–111.6	100.3	94.9–107.8
	Arithmetic mean	90% CI	Point estimate	90% CI
T_{max} individual difference	1.2	−0.6–3.0	0.25	−0.5–1.2

complained of diarrhea 2 h after first dosing (Viramune). No other adverse effects were reported or observed. All biochemical parameters showed no clinically relevant alterations.

Table 2 gives the values for $AUC_{(0-last)}$, $AUC_{(0-\infty)}$, C_{max}, K_e, $T_{1/2}$ and T_{max}. Table 3 summarizes the bioequivalence analysis of $AUC_{(0-last)}$, $AUC_{(0-\infty)}$, C_{max} and individual difference of T_{max} for NVP formulations. No period effect was observed for the pharmacokinetic parameters studied (data not shown).

Stability

The tests performed indicated that there was no significant degradation under the conditions described above.

DISCUSSION

The use of nevirapine has been associated with some serious adverse reactions such as hepatitis,[10,11] rash, Stevens–Johnson syndrome[12–14] and lipodystrophy.[15] However, these kinds of reactions were associated only with multiple-dose therapy and were not observed in this study.

The choice of dibenzepine as the IS for nevirapine was based on the presence of similar functional groups in both structures in addition to their similarity concerning M_r, elemental compositions and chemical behavior. Although deuterium-labeled isotopes are better internal standards than structural analogues, they are seldom commercially available and very expensive to synthesize, which apply to deuterated nevirapine.

Most of the assays reported for NVP determination are based on HPLC coupled with UV detection. The LOQ ranged from 25 to 400 ng ml^{-1}.[16,18–24] Retention times varied from 2.0 min to 39 min.[6,18–24] Sample preparation varied from simple protein precipitation[19,21,22] to liquid–liquid[23,24] and solid-phase extraction.[16–18,20]

Liquid chromatography coupled with mass spectrometry (LC/MS) using ion-trap technology has also been reported;[9] however the method employs a more complex and time-consuming liquid–liquid extraction (centrifugation for 5 min, freezing of the aqueous phase in a cryogenic bath, etc.). Although simple protein precipitation has been reported for nevirapine quantitation (see above), these methods do not have the necessary sensitivity for bioequivalence studies (1% of $C_{max} \approx 20$ ng ml^{-1}).

CONCLUSION

The method here described has several advantages. It has the lowest LOQ, employs inexpensive liquid–liquid extraction, has a fast run time and presents higher specificity since it employs MS/MS. Our results from a single-dose bioequivalence trial in healthy volunteers demonstrate that the method is adequate and reliable for such studies.

Nevirapine peak plasma concentrations and the T_{max} of both oral formulations were similar to those reported in the literature.[16] Since the 90% CI for C_{max}, $AUC_{(0-last)}$ and $AUC_{(0-\infty)}$ mean ratios are within the 80–125% interval proposed by the US FDA, it is concluded that the Nevirapina formulation provided by Far-Manguinhos, Brazil (200 mg tablet) is bioequivalent to Viramune (200 mg tablet) supplied by Boehringer Ingelheim Pharmaceuticals with regards to both extent and rate of absorption.

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