

Bioequivalence of Acenocoumarol in Chilean Volunteers: an Open, Randomized, Double-Blind, Single-Dose, 2-Period, and 2-Sequence Crossover Study for 2 Oral Formulations

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Key words

- therapeutic equivalence
- pharmacokinetics
- pharmaceuticals
- anticoagulant
- drug regulation

received 22.03.2012

accepted 26.05.2012

Bibliography

DOI <http://dx.doi.org/10.1055/s-0032-1316290>
 Published online: 2012
 Arzneimittelforschung
 © Georg Thieme Verlag KG
 Stuttgart · New York
 ISSN 0004-4172

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Abstract

The aim of this study was to compare the bioavailability of an oral formulation of the coumarin derivative-vitamine K antagonist acenocoumarol (Acebron™ 4 mg, Test) with the reference formulation (Neo-Sintrom™ 4 mg). We performed a single-dose, double-blind, fasting, 2-period, 2-sequence, crossover study design. Plasma concentrations of acenocoumarol were determined using a validated UPLC-MS/MS method. 24 healthy Chilean volunteers (11 male, 13 female) were enrolled and all of them completed the study. Adverse events were monitored throughout the study. The values of the pharmacokinetic parameters were (mean ± SD): $AUC_{0-24} = 1364.38 \pm 499.26$ ngxh/mL for the test and 1328.39 ± 429.20 ngxh/mL for the reference; $AUC_{0-\infty} = 1786.00 \pm 732.85$ ngxh/mL for the test

and 1706.71 ± 599.66 ngxh/mL for the reference; $C_{max} = 180.69 \pm 35.11$ ng/mL with a T_{max} of 1.83 ± 0.95 h for the test and 186.97 ± 38.21 ng/mL with a T_{max} of 2.19 ± 0.83 h for the reference. Regarding half life measurements, the mean ± SD of $t_{1/2}$ were 11.84 ± 4.54 h for the test and 11.08 ± 3.28 h for the reference. The 90% confidence intervals for the test/reference ratio using logarithmic transformed data were 97.89–100.87%, 98.62–101.99% and 98.64–102.38% for C_{max} , $AUC_{0-t(24)}$ and $AUC_{0-\infty}$. There were no significant differences in pharmacokinetic parameters between groups.

The results obtained in this study lead us to conclude, based on FDA criteria, that the test acenocoumarol formulation (Acebron™, 4 mg tablets) is bioequivalent to the reference product (Neo-Sintrom™, 4 mg tablets).

Introduction

Acenocoumarol is an anticoagulant drug that inhibits the action of vitamin K on the gamma-carboxylation of certain glutamic acid molecules, located in the coagulation factors II (prothrombin), VII, IX, X and protein C and S in the liver. The result of this inhibition is that no blood clotting can be triggered. The drug prolongs the thromboplastin-time test from about 36 to about 72 h, depending on the initial dosage; the time is normalized within a few days of drug withdrawal [1,2]. Oral administration of anticoagulant drugs is one of the most employed therapies in clinical practice for prevention and treatment of patients with arterial and venous thrombosis [3]. Acenocoumarol dosage usually is ranged from 8 to 12 milligrams on day 1 of therapy, on day 2, 4–8 milligrams is given. Subsequent doses are based on prothrombin time response, and have usually ranged from 1 to 8 milligrams daily [4]. After ingestion, Acenocoumarol is rapidly

absorbed with a systemic bioavailability of 60% minimum. The C_{max} is reached within 1–3 h after the dose and AUC values of plasma concentrations versus time are proportional to the administered dose in the range of 8–16 mg [5,6]. While in circulation, most of the drug remains bound to plasma proteins (98.7%), especially to albumin [7]. Acenocoumarol is metabolized primarily by the enzyme CYP2C9 and secondly by CYP1A2 and CYP2C19. The plasma half-life is 6.29 to 14.22 [1,2,5,6,8]. The R(+) enantiomer has higher in vivo anticoagulant activity due to its lower total plasma clearance in comparison with the S(-) enantiomer [1]. About 0.12–0.18% of the dose is excreted unchanged in urine. The cumulative excretion of metabolites and unchanged active substance for a period of 8 days, reach 60% of the dose in urine and 29% of the dose in the faeces [5,6]. Due to the large inter-individual variation associated with genetic modification, it is not possible to obtain a reliable correlation of plasma concentrations of Acenocoumarol and the appar-



ent level of prothrombin. In this respect, genetic variability associated with CYP2C9 accounts for 14% of the variability in the pharmacological response to the drug [1,2]. Importantly, patients older than 70 years tend to have higher plasma concentrations, compared with young ones, when given the same daily dose [2]. A literature search does not show bioequivalence studies on this drug, only biotransformation and pharmacokinetic research were found [1,2].

Given the pharmacokinetic and pharmacodynamic of the drug and its importance in the treatment of diseases associated with blood clotting, it is necessary to ensure quality combined with safety and effectiveness of similar drugs for solid dosage forms from multiple sources. Comparable drugs should be subjected to bioequivalence studies to demonstrate that they are interchangeable with the original product. This ensures that the exchange between 2 pharmaceutical equivalents did not cause alterations in the plasma concentration at steady state, attributable to the formulation [9–11].

The objective of the study was to evaluate, the bioequivalence of the oral formulation of acenocoumarol, in the pharmaceutical form of 4 mg tablets, ACEBRON™ (Recalcine Laboratories S.A, Santiago, Chile), in relation to the reference formulation NEO – SINTROM™ (Novartis Chile S.A, Santiago, Chile), after a single dose, taken by a group of 24 healthy volunteers.

Subjects and Methods



General Reagents and Medications

The medication used were: Test: tablets of 4 mg of acenocoumarol, ACEBRON™ (lot N° G-08644; expiration date 07/2010; Recalcine Laboratories S.A, Santiago, Chile) and Reference: tablets of 4 mg of acenocoumarol, NEO – SINTROM™ (lot N° 362901-CL 09; expiration date 09/2011; Novartis Chile S.A, Santiago, Chile).

Acetonitrile (HPLC grade) and other chemical reagents (analytical grade) were also purchased from Merck S.A. Chemicals Chile.

Inclusion and Exclusion Criteria

A group of healthy Chilean males and females aged 18–55 years with body mass index from 19 to 30 kg/m², were enrolled in this study. All volunteers provided written informed consent prior to study initiation. The details and purpose of the study were explained to all volunteers. All subjects were nonsmokers and without a history of alcohol or drug use.

All individuals were screened for suitability by an extensive review of their medical history, physical examination such as blood pressure and pulse, and interpretation of standard biochemical analyses (i.e., glucose, blood urea nitrogen, prothrombin time/International Normalized Ratio, creatinine, total protein, albumin, total bilirubin, alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase, drugs of abuse). Exclusion criteria included also HVI, hepatitis B or hepatitis C infection; consumption of any prescribed or over-the-counter drugs within 60 days before the study; or participation in a similar study within the past 90 days. Female subjects were not pregnant, planning to become pregnant or breast-feeding at the time of the study and were required to use an effective method of contraception throughout the study.

Study Design and Clinical Protocol

This was a single-dose, double-blind, fasting, 2-period, 2-sequence, crossover study design and blind bioequivalence study. The clinical protocol and informed consent for the study were reviewed and accepted by the Ethics Committee of Faculty of Medicine of the University of Chile.

Participants arrived at the study site the day before the beginning of the study at 9 PM and had an evening meal before 9.30 PM. After 11-h overnight fast, subjects received a single oral dose of either the test or reference formulation of acenocoumarol 4-mg tablets with 250 mL of water. Water was allowed as desired except for 1 h before and 2 h after drug administration. There was a 7-day washout (more than 5 times $t_{1/2}$ of the acenocoumarol) between 2 periods of treatments and the all the procedures were according to the declaration of Helsinki [12]. Randomization was performed using a random number table. The test-reference and reference-test sequences were balanced. Only the laboratory staff was blinded to treatment sequence. Subjects were asked if they suffered from undesirable effects such as nausea, vomiting, dizziness, and headache. The procedures performed to the volunteers were made taking into consideration international agreements regarding the study of drugs in humans and good clinical practices according to the criteria of the FDA [13].

Blood Sampling

Blood samples (5 mL) were collected into a tube containing 100 µL of sodium heparin (1.2 UI/µL) at 0 time (before administration), and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 8, 11 and 24 h (3 times $t_{1/2}$ of the acenocoumarol) after drug administration. Plasma was obtained by centrifugation at 2000 rpm for 10 min at 5°C. Plasma was subsequently divided into 2 aliquots which were stored as a sample and counter-sample at –20°C until analyzed.

Plasma Sample Preparation and Quantification of Acenocoumarol

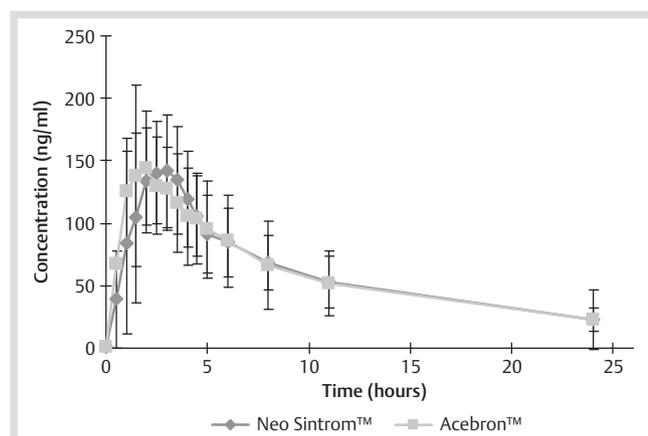
To an aliquot of 1 mL plasma sample in a glass tube, 20 µL of internal standard (Warfarin, 100 ng/mL) and 20 µL acetic acid 10% were added, then vortex-mixed briefly and extracted with 4 mL of ethyl acetate 100% by vortex-mixing for 10 min. After centrifugation at 3,600 rpm for 10 min, 2 mL of the organic layer were transferred into another glass tube and evaporated to dryness at 50°C under a gentle stream of nitrogen. The residue was reconstituted with 200 µL of the mobile phase by vortex-mixing for 30 s, then it was transferred to an autosampler vial and 10 µL were taken and injected into the UPLC-MS/MS system.

Plasma concentrations of acenocoumarol were determined using a new validated UPLC-MS/MS method which is an adaptation of those published by Huang et al. [14] for detection of warfarin in human plasma. Considering that warfarin has a similar chemical structure to acenocoumarol we decide to use it as internal standard. UPLC analyses were performed using an ACQUITY™ UPLC (Ultra Performance LC system, Waters, Milford, MA, USA.). UPLC separation was achieved using an Acquity UPLC™ bridged ethylene hybrid (BEH) HILIC C₁₈ column (2.1 mm × 50 mm, i. d., 1.7 µm). Determination was performed using a Micromass Quattro triple quadrupole spectrometry detector from Waters. The mobile phase consisted of ammonia acetate adjusted to pH 6.3: Acetonitrile (5:95 v/v). The detector was operated in the negative ionization and the multiple reaction monitoring (MRM) transitions monitoring were m/z 352.06 > 161.07 and m/z 307.0 > 106.0 for acenocoumarol and internal standard, respectively.



Table 1 Summary of the demographic data of the studied volunteers.

Characteristics	Value
Gender	
Male	11
Female	13
Age	
Mean (SD)	25 (6)
Range	18–30
Weight, kg	
Mean (SD)	66.1 (8.9)
Range	49.8–82.8
Body mass index, kg/m²	
Mean (SD)	23.4 (1.9)
Range	20.01–27.18

**Fig. 1** Mean plasma concentration-time profiles of Acebron™ (test) and Neo Sintrom™ (reference) 4-mg acenocoumarol tablets after single-dose administration in 24 healthy volunteers.**Table 2** Comparison of pharmacokinetic parameters of Acebron™ (test) and Neo Sintrom™ (reference) [4]-mg acenocoumarol tablets after single-dose administration in healthy volunteers.

Parameters	Test	Reference
AUC_{0-t} ng/ml *h-1		
Mean (SD)	1364.38 (499.26)	1328.39 (429.20)
% CV	36.59	32.31
$AUC_{0-\infty}$ ng/ml *h-1		
Mean (SD)	1786.00 (732.85)	1706.71 (599.66)
% CV	41.03	35.14
C_{max} ng/ml		
Mean (SD)	180.69 (35.11)	186.97 (38.21)
% CV	19.43	20.43
T_{max} , h		
Mean (SD)	1.83 (0.95)	2.19 (0.83)
% CV	51.60	38.08
$t_{1/2}$, h		
Mean (SD)	11.84(4.54)	11.08 (3.28)
% CV	38.34	29.57

CV: coefficient of variation between subjects; AUC: area under the curve; C_{max} : maximum concentration; T_{max} : time to reach the maximum concentration; $t_{1/2}$: half life

Pharmacokinetics and Statistical Analysis

Individual plasma concentration-time curves were constructed. The maximum plasma concentration (C_{max}) and time were obtained directly from these curves. The elimination rate constant (K_e), half-life ($t_{1/2}$), area under the plasma concentration-time curve AUC_{0-t} and $AUC_{0-\infty}$ were calculated by using pK

examine from STATA 10.0 software [15]. Plasma concentrations of acenocoumarol were plotted linearly and semi-logarithmically. FDA requirements were adopted for demonstrating bioequivalence between both formulations [16]. The bioequivalence could be concluded when the 90% confidence interval (CI) for the test/reference ratio of the means of the L_n -transformed AUC_{0-t} , $AUC_{0-\infty}$ and C_{max} of the 2 products were within 80% and 125% [17, 18]. The pharmacokinetic analyses were performed with “pK” routines to biopharmaceutical data with STATA v.10.0 software. We performed an analysis of variance (ANOVA) with *pkcross* proceeding and finally we did a bioequivalence test with *pkequiv* using classical interval and Schuirmann’s 2-one sided tests [15, 19]. Differences were considered significant at $p < 0.05$. FDA recommendations were adopted for the design of the study and pharmacokinetic and statistical analysis [16, 18, 20].

Results

The bioequivalence study was conducted in 24 healthy volunteers (11 male, 13 female). Their mean (SD) age was 25 (6) years (range, 18–30 years), their mean weight was 66.1 (8.9) kg (range, 49.8–82.8 kg), and their mean BMI was 23.4 (1.9) kg/m² (range, 20.01–27.18 kg/m²) (Table 1).

Adverse events were reported; for the reference formulation: 1 event of severe headache, 7 events of mild headache and 1 event of slight edema in the hand; and for the test formulation: 1 event of severe headache, 1 event of moderate headache, 5 events of mild headache and 1 event of mild heartburn.

Method validation

The mean \pm SD extraction recovery levels of acenocoumarol determined at 1.5, 65, and 130 ng/ml were 96.15% (0.1%), 112.50% (2.46%) and 93.87% (2.42%), respectively. LOQ for acenocoumarol was 0.5 ng/mL. Linearity was achieved at a concentration range from 0.5 to 400 ng/ml, with a typical equation for the calibration curve being $y = 0.5615x + 0.6019$ ($r > 0.999$). Intra-day accuracy determined at 1.5, 65, and 130 ng/mL were 101.56%, 106.03% and 95.87%, respectively. Intra-day precision determined at 1.5, 65, and 130 ng/mL were 10.34, 6.65 and 6.19% respectively. Inter-day precision (mean \pm SD) determined at 1.5, 65, and 130 ng/ml were 3.77, 3.93 and 5.34% respectively.

Pharmacokinetics of Acenocoumarol

The mean plasma concentration-time curves of acenocoumarol after single-dose administration of a 4-mg tablet of both products are presented in the Fig. 1, and the comparison of pharmacokinetics parameters between the 2 formulations are shown in Table 2. AUC_{0-24} (mean \pm SD) was 1364.38 ± 499.26 ng \times h/mL for the test product and 1328.39 ± 429.20 ng \times h/mL for the reference product. $AUC_{0-\infty}$ (mean \pm SD) was 1786.00 ± 732.85 ng \times h/mL for the test product and $1,706.71 \pm 599.66$ ng \times h/mL for the reference product. C_{max} (mean \pm SD) of the test product was 180.69 ± 35.11 ng/mL with a T_{max} of 1.83 ± 0.95 h and for the reference product was 186.97 ± 38.21 ng/mL with a T_{max} of 2.19 ± 0.83 h. Finally, $t_{1/2}$ (mean \pm SD) was 11.84 ± 4.54 h for the test product and 11.08 ± 3.28 h for the reference product. According to the mean plasma levels of the 24 subjects completing the study, the relative bioavailability ratio of test/reference was 1.078 on the basis of AUC_{0-24} .



Table 3 Analysis of variance (ANOVA) for a 2×2 crossover study to C_{max} , AUC_{0-t} and $AUC_{0-\infty}$ to Acebron™ (test) and Neo Sintrom™ (reference) [4]-mg acenocoumarol tablets after single-dose administration in healthy volunteers.

Source of Variation	Partial SS	Df	MS	F	p-value
C_{max}					
Inter subjects					
Sequence effect	0.02	1	0.02	0.40	0.5357
Residuals	1.33	22	0.06	2.47	0.0195
Intra subjects					
Treatment effect	0.01	1	0.01	0.50	0.4852
Period effect	0.06	1	0.06	2.45	0.1318
Residual	0.54	22	0.02		
AUC_{0-24h}					
Inter subjects					
Sequence effect	0.46	1	0.46	3.13	0.0908
Residuals	3.25	22	0.15	2.49	0.0186
Intra subjects					
Treatment effect	0.01	1	0.01	0.10	0.7585
Period effect	0.04	1	0.04	0.63	0.4367
Residual	1.30	22	0.06		
$AUC_{0-\infty}$					
Inter subjects					
Sequence effect	0.36	1	0.36	2.26	0.1467
Residuals	3.52	22	0.16	2.06	0.0492
Intra subjects					
Treatment effect	0.02	1	0.02	0.22	0.6413
Period effect	0.20	1	0.20	2.58	0.1223
Residual	1.71	22	0.08		

SS: Square Sum; Df: Degree of freedom; MS: Mean Square; F: Fisher

Table 4 Bioequivalence test (Schuirmann's test) to C_{max} , AUC_{0-t} and $AUC_{0-\infty}$ to Acebron™ (test) and Neo Sintrom™ (reference) [4]-mg acenocoumarol tablets after single-dose administration in healthy volunteers.

N=24	90% CI**		Schuirmann's test		
	test limits		Two	One Side Test	p-value
Ln* C_{max}					
difference	-0.110	0.046	upper	-23.764	<0.001
Rate	97.894%	100.874%	lower	22.344	<0.001
Ln AUC_{0-24h}					
difference	-0.099	0.142	upper	-20.030	<0.001
Rate	98.618%	101.994%	lower	20.652	<0.001
Ln $AUC_{0-\infty}$					
difference	-0.100	0.176	upper	-17.869	<0.001
Rate	98.643%	102.388%	lower	18.814	<0.001

Ln: Natural Logarithm; ** Confidence Interval

Bioequivalence Analysis of Acenocoumarol

ANOVA test of the pharmacokinetic parameters is shown in **Table 3**. 90% confidence intervals for the test/reference ratio using logarithmic transformed data were 97.89–100.87%, 98.62–101.99%, 98.64–102.38% for C_{max} , AUC_{0-24} and $AUC_{0-\infty}$, respectively. The probability of exceeding the limits of acceptance (Schuirmann's 2 one-side test), the p-value for the probability of bioequivalence are also shown in **Table 4**.

Discussion

Bioequivalence studies make available good quality and cheaper drugs to the market contributing to a more rational use of economic resources [21]. Multisource formulations containing acen-

ocoumarol require, according Chilean guidelines, studies to demonstrate therapeutic equivalence [22]. Therefore, we address this study to assess this issue.

We did not study acenocoumarol enantiomers because measurement of individual enantiomers, in BE studies, is recommended only when all of the following conditions are met: (a) the enantiomers exhibit different pharmacodynamic characteristics, (b) the enantiomers exhibit different pharmacokinetic characteristics, (c) primary efficacy and safety activity resides with the minor enantiomer, and (d) nonlinear absorption is present [17]. Therefore, even though acenocoumarol enantiomers have different pharmacokinetic and pharmacodynamic characteristics [23,24] and the racemic mixture contain equal parts of R(+) and S(-) enantiomers [23], the primary efficacy and safety activity resides in the R(+) enantiomer which is found in larger amount in serum. The plasma clearance of the S(-) enantiomer is 10-fold higher than the R(+) enantiomer. Therefore anticoagulation mainly relies on R(+) enantiomer despite a higher intrinsic potency of the S(-) enantiomer [1,24,25]. According to that, we concluded it is appropriate to measure concentrations of acenocoumarol in plasma using a non-enantiomer-specific assay.

In the present study, when pharmacokinetics parameters were analyzed using ANOVA test, non statistically significant differences were observed between both formulations in the logarithmically transformed AUC or C_{max} values. The results show that the confidence intervals of log-transformed ratios for C_{max} and AUC with both formulations were within the predetermined equivalence range of 80% and 125% [17,18]. Schuirmann's test indicated similar results with $p < 0.05$ for tested hypotheses.

The adverse events reported were dealt without difficulty and they were treated without drugs.

Up to our knowledge, based on scientific literature, there are reports of several methods for determination of acenocoumarol described [26–28], but none of them use UPLC-MS/MS methodology and no bioequivalence studies on this compound have been previously published. Therefore, the present study shows a new rapid and sensitive UPLC-MS/MS method for determination of acenocoumarol in plasma for bioequivalence studies. This method was validated according to the FDA recommendations [29].

Our study have some limitations, particularly because the data were obtained from healthy Chileans subjects who received a single dose of acenocoumarol, therefore the pharmacokinetic characteristics should differ from patients who receive multiple doses and from other non Chileans mestizo population because of different genetic background. Despite this, we believe it is a very good reference for similar Latin American populations. Other limitation of this study could be the use of a single dose from 4 mg tablet of acenocoumarol, considering that AUC values of plasma concentrations *versus* time are proportional to the administered dose in a higher range [8–16mg].

Finally, similar to other oral anticoagulants, acenocoumarol exhibits a narrow therapeutic index [30], therefore we recommend that any patient who switches between reference product and generic formulation should be carefully monitored according to the International Normalized Ratio (INR) [31].

Conclusion

The results of this study suggest that the test 4-mg acenocoumarol tablet (Acebron™) is bioequivalent and interchangeable

with the reference product (Neo-Sintrom™). We did not find any statistically significant difference in C_{max} or AUC values between test and reference formulations of 4-mg acenocoumarol tablets in our group of healthy volunteers.

Acknowledgements

This manuscript is dedicated to the memory of Mr. Santiago Leyton M. an excellent technician and very good friend who left us unexpectedly. The authors wish to thank to Mrs. Magdalena Orellana, Ms. Jeannette Hermosilla, Mr. Héctor Valenzuela, Mr. Ramiro Pérez, and Mr. Juan Rojas for their excellent technical support.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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