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Determination of Therapeutic Equivalence (TE) for 5 Generic Products (GP) of Penicillin G (PEN) Using the Neutropenic Murine Thigh Infection Model (NMTIM)

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ABSTRACT

Background: legal use of GP in human and veterinary medicine have great impact in price regulation worldwide, but numbers of GP makers are increasing overwhelmingly. Beyond quality problems, concern is growing about the scientific basis of using bioequivalence (BE) as surrogate proof of TE, the accepted dogma. To test it, bactericidal efficacy of GP of PEN made in Colombia was compared in vitro and in vivo against the original compound (OC) in simultaneous experiments, employing the same methods required for new antimicrobials. **Methods:** microbiologic assays with *S. aureus* ATCC 6538p and broth micro dilution minimal inhibitory and bactericidal concentrations (MIC/MBC) were used to determine BE by comparison of standard curves (SC) and in vitro activity. The NMTIM used 6 week-old specific pathogen free ICR:CD-1 female mice weighting 25±2 g infected with *S. aureus* GRP-0057, a PEN-susceptible clinical isolate. The sigmoid dose-response model was applied to calculate by least squares nonlinear regression (NLR) pharmacodynamic parameters (PDP) like Emax and bacteriostatic dose (BD). SC were compared by linear curve fitting analysis (CFA), whole NLR by nonlinear CFA, individual PDP by nonlinear CFA and Wilcox on-Mann-Whitney test (WMW). MIC/MBC by WMW. **Results:** all 5 GP were made in Colombia; 1 had greater amounts of PEN (intercept = 0.0052 vs. 0.0041 mg/l, P<0.0001), but did not differ in MIC/MBC from the OC. Only 1 of the other 4 GP had lower MIC/MBC, 0.01/0.02 vs. 0.07/0.10 mg/l for the OC (P = 0.013). Mice had $10^{6.4\text{-}7.1}$ CFU/thigh (CFU/g) when treated with PEN 0.094-24 mg/kg for 24 hours (24h) divided q1h. At the end of therapy, untreated controls had $10^{7.65\text{-}7.93}$ CFU/g (24h growth = 2.92-3.33 $\log_{10}$ CFU/g). All 5 GP failed TE: Emax = 2.01-3.26 vs. 4.87 $\log_{10}$ CFU/g, and BD = 6.94-120.1 vs. 1.65 mg/kg/24h for GP and OC respectively (P<0.0005). **Conclusions:** GP of PEN do not exhibit TE with the OC. Current criteria for BE and TE deserve review.

INTRODUCTION

Generic medicines are important worldwide for price control and the only access to treatment for people in poor nations. Their market reaches 23 million dollars a year in United States alone, and comprises a significant portion of exports economy for many countries. The World Health Organization and virtually all regulatory agencies support industrialization and commercialization of generics, although enforcement of good laboratory / manufacturing practices and quality control and are as diverse as the cultures by them represented. This way, the quality of generics has become a source of permanent concern, but not so therapeutic equivalence, which requires no prove. Under such assumption, regulatory agencies expect the same therapeutic efficacy if generics demonstrate pharmaceutical equivalence with the comparator, often with little regard for the complexity of clinical and molecular pharmacology.

Different to other illness, the treatment of infectious diseases involves three key elements: the host cell, the drug, and the microorganism, constituting perhaps the best example for complex pharmacology. In spite of this fact, regulatory agencies do not classify antimicrobials in the "narrow therapeutic range" category. This approach could neglect important differences in pharmacology, physiology and microbiology, because it considers any generic antibiotic with 80-125% variation in pharmacokinetic profile equivalent to the original compound to treat vastly distinct conditions and patients, such as lower urinary tract infections and bacterial meningitis.

The goal of this study was to determine therapeutic efficacy of all generic penicillin G products licensed for human use in Colombia using the animal model of infection. To confirm pharmaceutical and therapeutic equivalence, we compared generics with the original compound in terms of amount and potency of active principle, in vitro susceptibility tests, and in vivo bactericidal efficacy.

MATERIALS AND METHODS

Infecting organism and in vitro susceptibility tests: we bought all penicillin products from local drugstores and dissolved them as indicated by the maker. The concentration of each product was not adjusted, even if experimental data demonstrated lack of pharmaceutical equivalence. *Staphylococcus aureus* GRP-0057, a wild-type clinical strain isolated in blood cultures from a child with community-acquired bacteremia, was used to infect the mice. Besides standard clinical susceptibility tests for all proper antibacterials (Vitek 2®, Biomeriéux, Durham, NC), we determined the MIC and MBC of each generic and the original penicillin G product by broth microdilution, following NCCLS criteria [3]. Statistical significance of any difference between geometric means (2-3 assays, each by duplicate) was calculated with the Kruskal-Wallis test (KW), followed by the Wilcoxon-Mann-Whitney test (WMW) to identify the generic(s) responsible for such difference (SPSS 11®, SPSS Inc., Chicago, IL).

Microbiologic assays: we used an USP standard microbiologic assay, with Difco™ Antibiotic Medium 1 as diffusion agar and *S. aureus* ATCC 6538p as testing strain, to determine potency and concentration of active principle [4]. Assays were done simultaneously in a custom-designed glass plate, large enough to accommodate duplicates of 10 physiological concentrations of all available products (0.0018-128 mg/l), using the original compound as the reference penicillin G. The same researcher measured zone-sizes for all assays using an electronic caliper (Mitutoyo Corp., Kawasaki, Japan). Linear regressions of the results produced standard curves, which intercepts and slopes were compared by Curve Fitting Analysis (Prism 4®, GraphPad Software Inc., San Diego, CA). The finding of similar slopes (parallel standard curves) with different intercepts implied comparable potency but different concentration of the active principle, so we calculated penicillin G concentration as an exact percentage with respect to the original compound. On the other hand, divergent standard curves (different slopes) implied different potency at all concentrations of the active principle, in which case we expressed potency of generic penicillins as a range with respect to the potency of the original compound (100%), calculating it at the lowest and highest concentrations measured in the microbiologic assays.

Determination of therapeutic efficacy with the neutropenic murine thigh infection model (NMTIM): to find out if generic penicillins had therapeutic equivalence with the original compound, we selected the same animal model used to study new compounds and to make a revolution in pharmacokinetics (PK) and pharmacodinamics (PD) of antimicrobials [5]. We founded our own colony of outbred Swiss Albine mice from imported parents (Harlan, Indianapolis, IN), and called it strain Udea:ICR(CD-1) [6]. Animals are confined, grown and manipulated within high technology microbiological barriers (Super Mouse Micro-Isolator® System, Lab Products Inc., Seaford, DE). The protocol for mice welfare and breeding conditions follows the Guide [7]. An independent party (Taconic Anmed, Rockville, MD) certified their murine pathogen free (MPF) status. *Staphylococcus aureus* GRP-0057 was grown in Trypticase Soy Broth (TSB, Becton-Dickinson, Sparks, MD) to obtain log-phase cultures for mice inoculation, and on solid media (TSA) for colony counting. Broth cultures were adjusted to 10^8 $\log_{10}$ CFU/ml before inoculating 0.1 ml into each thigh.

MATERIALS AND METHODS (CONT...)

The NMTIM is well described elsewhere [8]. Briefly, 6 week-old female mice weighting 25±2 g were rendered neutropenic (zero neutrophils per µl) by intraperitoneal injections of cyclophosphamide 4 days (150 mg/kg) and 1 day (100 mg/kg) before infection. Two animals were infected for each of five doses that went from minimal (2.34) to maximum (24 mg/kg/24h) antibacterial effect, totaling 10 mice per penicillin G product. Penicillin was injected SQ every one hour (h) in 0.2 ml volumes, starting 2 h after infection and continuing for 24 h, when all treated animals were sacrificed. All experiments included the original compound and at least one generic product, as well as 6 infected but untreated control mice that were humanely euthanized in pairs at the time of infection (-2h), and at the time of starting (0h) and finishing (24h) treatment in the experimental groups. Then, the thighs were dissected under aseptic conditions, homogenized by separate, properly diluted, plated by duplicates on solid media (TSA), and incubated overnight under air atmosphere. Manual colony counting preceded data registry and analysis. To determine net antibacterial effect, the number of CFU remaining in the thighs after 24 h of penicillin treatment was subtracted from the number of CFU that grew in the thighs of control mice during the same period (24h control CFU/thigh minus 0h control CFU/thigh). This way, net bacterial death or growth produces negative or positive values, respectively. For this model, one thigh weighs one gram, and the limit of detection is 100 CFU/g; data was registered as $\log_{10}$ CFU/g.

For statistical analysis and characterization of in vivo antimicrobial efficacy of each product, we applied the sigmoid dose-response model with the Hill equation, and calculated PD parameters (PDP) by least squares nonlinear regression (SigmaPlot 8.0®, SPSS Inc., Chicago, IL). Primary PDP included maximum effect (Emax), effective dose reaching 50% of Emax (ED_{50}), and slope (N). From these, we calculated the secondary parameters bacteriostatic dose (BD) and the dose needed to kill the first (1LKD) and second (2LKD) $\log_{10}$ CFU/g in 24h. To compare antibacterial efficacy of each generic against the original compound, we employed CFA of their nonlinear regressions under the null hypothesis that the data for both compounds came from the same population (i.e., no difference in effect between generic and innovator) (Prism 4®, GraphPad Software Inc., San Diego, CA). In case of violation of normality and parametric assumptions, the non-parametric tests KW followed by Permutation One-Way ANOVA with General Scores (POWAGS) were applied to raw data. If significant differences were found between all generics and the innovator compound, the WMW test was applied to each dataset (generic versus original) to identify the product responsible for such difference (StatXact-5®, Cytel Software Corp., Cambridge, MA). Exact P values were calculated in all cases, and statistical significance accepted when two-sided P<0.05. To illustrate the potential clinical impact of the differences, figures compare primary PDP Emax ($\log_{10}$ CFU/g in 24h) and secondary parameters BD, 1LKD, and 2LKD (mg/kg/24h) of generics with the innovator product.

RESULTS

Table 1 lists some characteristics of the penicillin G generic products available in Colombia between 2001 and 2004. All generics were made in Colombia, although active principles are usually imported. These products were legal, widely distributed in drugstores, and their prices ranged between 25and 66% with respect to the original compound. We used code numbers to replace the manufacturers names.

Table 1. Characteristics of the penicillin G products included in the study

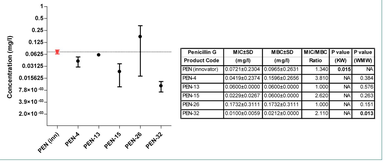
Penicillin G Product Code	Manufacturer	Country of Manufacture	Batch	Price Respect to Innovator (%)
PEN (innovator)	Bristol	Ecuador	ED247, EG049, EK149	
PEN-4	AZ Pharma	Colombia	050139, 090282, 030220	46
PEN-13	Formas Genéricas	Colombia	S1E1001	34
PEN-15	Genfar	Colombia	020501, 010402, 010200	40
PEN-26	Pentacoop	Colombia	20116, 21070	25
PEN-32	Recipe	Colombia	030225, 010201	66

In vitro susceptibility tests: five generic products were available at the time of these experiments; the original compound was the reference product in all experiments and comparisons. Except for PEN-32, there were no differences in MIC, MBC or MBC/MIC ratio against *S. aureus* GRP-0057. MIC and MBC of PEN-32 were 7.21 and 4.55 times lower than the original compound, respectively ($P = 0.013$).

.**Microbiological assays:** only four generic products were available at the time of these experiments; the original compound was the reference product in all experiments and comparisons. The coefficients of determination (r^2) of their standard curves were very high, ranging between 0.9355 and 0.9874. There was no difference in potency or concentration of the active principle for two generic products (PEN-15 and PEN-26). On the other hand, PEN-4 and PEN-32 had significant differences with the original compound in their intercepts, but not in their slopes, suggesting greater concentration of the active principle but similar potency (Figure 2).

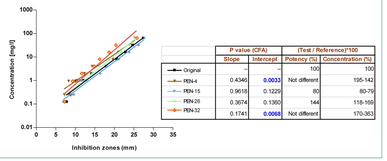
RESULTS (CONT...)

Figure 1. Geometric mean values for Minimal Inhibitory (MIC) and Bactericidal (MBC) Concentrations of six penicillin G products against *S. aureus* GRP-0057



Penicillin G Product Code	MIC±SD (mg/l)	MBC±SD (mg/l)	MIC/MBC Ratio	P value (KW)	P value (WMW)
PEN (innovator)	0.0721±0.2304	0.0965±0.2831	1.340	0.015	NA
PEN-4	0.0419±0.2374	0.1596±0.2656	3.810	NA	0.364
PEN-13	0.0600±0.0000	0.0600±0.0000	1.000	NA	0.515
PEN-15	0.0229±0.0267	0.0600±0.0000	2.620	NA	0.283
PEN-26	0.1732±0.3111	0.1732±0.3111	1.000	NA	0.151
PEN-32	0.5150±0.0058	0.3212±0.0000	2.110	NA	0.013

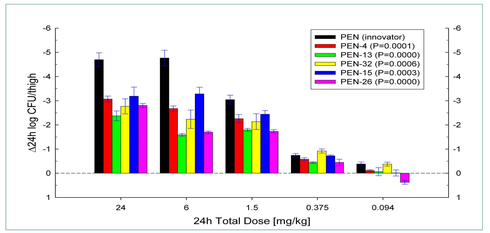
Figure 2. Standard curves of four generic products of penicillin G compared with the original compound. Microbiologic assay with DifcoTM Antibiotic Medium 1 as diffusion agar and S. aureus ATCC 6538p as test organism. Limit of detection: 0.0156 mg/l.



	P value (CFA)		(Test / Reference)*100	
Slope	Intercept	Potency (%)	Concentration (%)
Original		-	100	100
PEN-4	0.4340	0.0033	Not different	195-142
PEN-15	0.9618	0.1229	80	80-79
PEN-26	0.3674	0.1365	144	118-169
PEN-32	0.1741	0.0068	Not different	170-303

In vivo efficacy: five generic products were available at the time of these experiments; the original compound was the reference product in all experiments and comparisons. PEN-26 and PEN-32 gave poor nonlinear regressions (primary PD parameters ED_{50} and N not significantly different from zero), which indicated non-parametric analysis of the raw data. Dose by dose comparisons against the original compound produced significant differences taking all five generics as a group (P=0.0000, KW followed by POWAGS) and independently (all P≤0.0006, WMW, Figure 3). Given that the Emax obtained by nonlinear regression was significantly different from zero for all generics, and that Emax, ED_{50} and N were all significant for the original compound and three of five generics (PEN-4, PEN-13, PEN-15), we also analyzed the data with parametric statistics (CFA of the nonlinear regressions). Emax and BD of the original compound were 4.87 $\log_{10}$ CFU/g and 1.65 mg/kg/24h, respectively. PEN-4 and PEN-32, substandard generics because of their significantly greater concentrations of active principle had, unexpectedly, significantly lower in vivo efficacy, with Emax 1.93-2.86 logs smaller and BD 52-55 times greater than the original compound. Contrary to what is expected from generics with pharmaceutical equivalence, in vivo efficacy of the other three products (PEN-13, PEN-15, and PEN-26) was also significantly lower, with Emax 1.61-2.39 logs smaller and BD 4.18-72.7 times greater than those of the comparator. It was not possible to calculate 1LKD and 2LKD because none of the five generic products reached bactericidal effect (Figure 4). A comparison of the Emax obtained for all generics with that of the original compound illustrates the potential clinical significance of the difference in efficacy (Figure 5).

Figure 3. Non-parametric analysis to determine differences in efficacy of five generic products of penicillin G compared with the original compound. *Staphylococcus aureus* GRP-0057 in the neutropenic murine thigh infection model (dosing q1h by SQ injections)



RESULTS (CONT...)

Figure 4. Curve fitting analysis of dose-effect nonlinear regression to determine in vivo efficacy of five generic products of penicillin G against *S. aureus* GRP-0057, compared with the original compound in the neutropenic murine thigh infection model (dosing q1h by SQ injections)

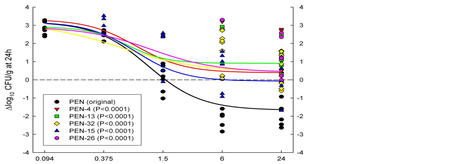
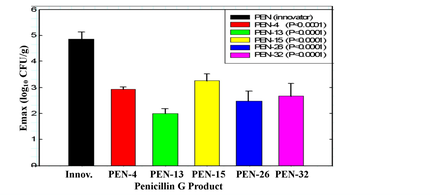


Figure 5. Emax comparison between five generic Penicillin G products and the original compound



CONCLUSIONS

- This is the first time that therapeutic equivalence is determined with an experimental approach. Compared with the original compound, all generic products of penicillin G failed in vivo against *S. aureus* GRP-0057, an invasive wild-type clinical strain.
- Therapeutic efficacy cannot be assumed, even from generics without significant differences with the original compound in terms of MIC, MBC, potency or concentration of active principle (pharmaceutical equivalency).
- Substandard generics with greater concentrations of penicillin G are also inferior in vivo, even when their doses were not adjusted to the actual concentrations. This finding implies that factors exclusively found in vivo attempt against the capacity of the generic product to eradicate the invading organism. The design of this study does not allow identification of such factors.
- In vitro susceptibility tests do not detect failures in therapeutic equivalence of generic penicillin G.
- The concepts of pharmaceutical equivalence, bioequivalence, and therapeutic equivalence must be re-assessed, because WHO and all regulatory agencies use the first two as surrogate criteria for the third.
- Pharmacokinetics and pharmacodynamics of generic antimicrobials must become an urgent priority for research, because quality control, good laboratory / manufacturing practices, and compliance with "bioequivalence" do not seem enough to guarantee therapeutic equivalence.

REFERENCES

1. Senn SJ. In the blood: proposed new requirements for registering generic drugs. Lancet 1998;352:85-6.
2. Food and Drug Administration (FDA); Center for Drug Evaluation and Research (CDER). Statistical approaches to establishing bioequivalence. Rockville, MD, USA. FDA; 2001. Available at http://www.fda.gov/cder/guidance/index.htm
3. National Committee for Clinical Laboratory Standards. 2004. Performance standards for antimicrobial susceptibility testing; fourteenth informational supplement. Vol. 24, No. 1, M100-S14. National Committee for Clinical Laboratory Standards, Wayne, PA.
4. Bennett JV, Brodie JL, Benner EJ, Kirby WMM. Simplified, accurate method for antibiotic assay of clinical specimens. Applied Microbiol 1966; 14:170-77.
5. Craig WA. Pharmacokinetic/pharmacodynamic parameters: rationale for antibacterial dosing of mice and men. Clin Infect Dis 1998; 26:1-10.
6. Zuluaga AF, Salazar BE, Galvis W, Loaiza SA, Agudelo M, Vesga O. Foundation of the first functional MPF animal facility in Colombia. Iatreia 2003;16 (2):115-131 (Spanish).
7. Institute of Laboratory Animal Resources; Commission of Life Sciences. Guide for the care and use of laboratory animals. National Research Council: 1996. National Academy Press, Washington, D.C., USA.
8. van Ogtrop ML, Andes D, Stamstad TJ, Conklin B, Weiss WJ, Craig WA, Vesga O. In vivo pharmacodynamic activities of two glycylcyclines (GAR-936 and WAY 152,288) against various Gram-positive and Gram-negative bacteria. Antimicrob Agents Chemother 2000;44:943-9.