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Novel methods to assess bioequivalence

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Importance of the field: The ultimate goal of bioequivalence is to assess the expected *in vivo* equivalence of two drug products of the same active moiety.
Areas covered in this review: In this review, we present the classic approach of bioequivalence assessment, some situations of special importance such as the role of metabolites and highly variable drugs, and the current regulatory state in North America and Europe. Special emphasis is given to the methods proposed for solving the problems caused by high variability such as multiple-dose studies, replicate designs, individual bioequivalence and the widening of bioequivalence limits. Other issues discussed include the concept of biowaivers and the rising field of the equivalence of biologicals (biosimilars).
What the reader will gain: The reader will gain an understanding of why bio-equivalence assessment is necessary, how it is performed and what one should be aware of when planning to conduct a bioequivalence study.
Take home message: The aim of bioequivalence studies is to ensure comparable *in vivo* performance of two drug products. This is accomplished by performing an appropriate clinical study which should be capable of ensuring the drug's safety and efficacy for consumers with less human exposure and costs of producing.

1. Introduction

Drug absorption through the gastrointestinal (GI) tract is a complex process affected by several factors, such as the physicochemical properties of the active substance, the GI physiology and the formulation properties of the drug product [1]. Before reaching the systemic circulation, drug molecules must cross several semipermeable cell membranes. The latter are biologic barriers which selectively inhibit passage of chemical compounds. Important physicochemical factors for drug absorption include the degree of ionization, aqueous solubility, lipophilicity, surface area, particle size and crystal form. Besides, formulation effects may also affect drug dissolution or release, which may have a significant impact on drug absorption process. Small changes in the formulation may result in significant differences in the observed plasma concentration–time profile.

Observations in the 1950s and 1960s raised the first indications of bioequivalence (BE) testing [2-7]. It was realized that the drug product's efficacy depends on the amount of the active moiety which becomes available at the site of action and how rapidly this process takes place. The term bioavailability was then officially introduced by the FDA to describe both the extent and rate of drug arrival in the systemic circulation, while bioequivalence refers to comparative bioavailability studies [8-10]. Since then, BE assessment relies on the assumption that the therapeutic effect is a function of concentration of the active moiety in the systemic circulation. The regulatory authorities worldwide consider two drug products to be

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Article highlights.

- Bioequivalence studies are used to assess the expected *in vivo* equivalence of two drug products of the same active moiety.
- Classically, bioequivalence assessment relies on the concept of average bioequivalence using a two-period, two-treatment, crossover 2 × 2 design.
- As variability increases it becomes too difficult to establish bioequivalence unless a large number of subjects are recruited.
- Several methodologies have been proposed to overcome the problems caused by high variability. Currently, regulatory authorities in North America and Europe suggest the use of semi-replicate designs in case of highly variable drugs.
- Biowaivers allow the use of evidence other than *in vivo* data.
- Demonstrating similarity of biologicals (biosimilars) requires a methodology different from that used for low molecular mass drugs.

This box summarizes key points contained in the article.

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bioequivalent if they contain the same active substance, are at the same molar dose, and their rate and extent of absorption are similar to ensure comparable *in vivo* performance [11,12]. During the first years of development of BE setting, physicians by general consensus (actually without scientific basis or clinical evidence) suggested that a difference of 20% between the two formulations would have no clinical significance for many drugs. This recommendation is now interpreted as a pre-defined allowable difference in the means of pharmacokinetic (PK) variables, namely, C_{max} and AUC.

2. Methods to assess BE

2.1 Background: classic BE approach

Classically, BE assessment relies on the concept of average BE [13]. Two drug products, namely, a product under evaluation (test, T) versus an innovator's product (reference, R), are considered bioequivalent if the calculated 90% CI for the difference of the logarithmic-transformed mean measures of bioavailability (i.e., μ_T (in-transformed mean values of the pharmacokinetic parameters for T) and μ_R (in-transformed mean values of the pharmacokinetic parameters for R), for the T and the R formulations, respectively) fall between pre-specified values for the upper and lower BE limits [13,14]. The average BE testing approach is based on the use of constant BE limits (BEL_0) defined by the regulatory authorities [11,12,14]. The criterion applied to the determination of BE is mathematically expressed with Equation 1:

$$(1)$$
$$-BEL_0 \leq \mu_T - \mu_R \leq BEL_0$$

where BEL_0 is usually set equal to $ln(1.25)$ (**Figure 1A**). Assuming the classic two-period, two-treatment, crossover 2×2 design, with equal numbers of subjects in each

sequence, the estimated upper and lower limits of the 90% CI are given by Equation 2 [15,16]:

$$(2)$$
$$Upper, Lower\ limits\ of\ the\ 90\%\ CI = \exp\big((m_T - m_R)$$
$$\pm t_{0.05,N-2}\sqrt{s^2 2/N}\big)$$

where m_T and m_R are the estimated log-transformed mean measures of bioavailability for T and R product, respectively. The term s is the mean square error of *ANOVA* which is considered to reflect the intra-subject variability (σ_W^2), t is the *t*-student statistic with *N-2* degrees of freedom and *N* is the number of subjects participating in the BE study.

However, the use of average BE approach exhibits several limitations as in the case of highly variable drugs (HVDs) or toxic drugs with low variability and narrow therapeutic range [16,17]. It has been realized that average BE allows large differences between the means for drug products with low residual variability. The latter constitutes a potential problem of switchability for multisource formulations, each declared bioequivalent to the same R product [16].

2.2 HVD

HVDs and drug products exhibit $\sigma_W^2 > 30\%$ in the bioavailability parameters [17,18]. σ_W^2 can be attributed to the drug substance itself and/or to the formulation characteristics. This definition of BE given in Equation 2 has the advantage of ensuring *a priori* the relative consumer risk. However, it becomes obvious from Equation 2 that as variability (*s*) increases it becomes too difficult to establish BE unless a large number of subjects are recruited. In order to overcome the obstacle of high variability several approaches have been proposed such as steady-state studies, replicate designs for single-dose studies, individual bioequivalence (IBE), widening of BE limits and the application of scaled BE methods.

2.2.1 Multiple-dose studies

It has been observed that the variability in the PK parameters is often lower at steady-state conditions than after single dosing [19-21]. For C_{max}, the reduced variation at steady-state can be attributed to its lower kinetic sensitivity in reflecting absorption rate [13,22]. In this vein, multiple-dose steady-state studies have been proposed to reduce σ_W^2 [19]. It has been highlighted that the 90% CIs were narrower after multiple- than single-dose studies [23]. However, regulatory authorities do not support the use of multiple dose BE studies as they may hide differences in absorption profiles between the two formulations.

2.2.2 Replicate designs

Replicate designs for single-dose studies have been proposed to reduce the required number of subjects in case of HVD [11,13,19,24]. Roughly speaking, the same statistical power can be attained using about 50% as many volunteers

in a four-period design as in a two-period study. Nevertheless, these studies increase the duration of exposure of the volunteers, cannot be applied to drugs with long half-lifes and may lead to increased occurrence of subject withdrawals.

2.2.3 IBE

IBE was introduced as an effort to consider σ_W^2 of T and R formulations [14,25-30]. The T-R differences of the bioavailability measures (e.g., AUC, C_{max}) are contrasted to the R-R differences using a replicate study design. The IBE approach takes into consideration not only the T-R differences, but also their σ_W^2 values and the variance component of the subject-by-formulation interaction (σ_D^2). Mathematically, IBE is summarized by Equation 3:

$$(3)$$

$$\left[(\mu_T - \mu_R)^2 + \left(\sigma_{WT}^2 - \sigma_{WR}^2 \right) + \sigma_D^2 \right] / \sigma^2 \le \theta_1$$

where ϑ_I is a regulatory criterion, σ_{WT}^2 and σ_{WR}^2 correspond to σ_W^2 of T and R formulations, respectively, while σ^2 is a variance term. The last is constant when σ_{WR}^2 is lower than a pre-specified value (σ_0^2) or it is set equal to σ_{WR}^2 when the latter exceeds the σ_0^2 value which is the case of HVD [14,27,31]. Even though IBE approach relies on an attractive concept, several practical problems have limited its application and it is not further used in BE practice [29].

2.2.4 Widening of BE limits to pre-fixed values

Another opportunity to face-off the high σ_W^2 encountered in BE studies is the widening of BE limits to arbitrary values. In case of C_{max}, it was proposed to widen BE limits to pre-specified constant values such as 0.75 – 1.33 or 0.70 – 1.43 and justify any safety/efficacy concerns [11]. This widening is suggested to take place only if σ_W^2 exceeds a pre-defined variability value (**Figure 1B**). However, this approach is less sensitive to detect differences between the T and R formulations in case of low or moderate variable drugs and may lead to unfair treatment when different formulations of the same active substance are evaluated in separate BE studies [32-34].

2.2.5 Scaled procedures

Scaled BE procedures reflect the need that BE criteria should be more liberal as variability increases. The basic feature of all scaling procedures relies on their gradual expansion with σ_W^2 [16,35,36]. Generally, the acceptance criterion of BE can be expressed as:

$$(4)$$

$$-k \cdot \sigma_W \le \mu_T - \mu_R \le k \cdot \sigma_W$$

where k is a proportionality constant. The term σ_W^2 strictly corresponds to the exact σ_W^2 of a specific drug which can only be calculated from a replicate design. However, in case of the classic 2×2 crossover design, σ_W^2 refers to the residual variability estimated from *ANOVA* analysis.

2.2.5.1 Scaled average bioequivalence

The regulatory criterion of BE described by Equation 4 can be transformed into Equation 5 leading to an approach known as scaled average bioequivalence (SABE) [37,38]. The latter is defined as:

$$(5)$$

$$-k \le \frac{\mu_T - \mu_R}{\sigma_W} \le k$$

The confidence limits for the SABE method can be calculated using a non-central t-distribution approach or a numerical approximation [38,39]. It should be emphasized that the model for the SABE approach can be converted to a scaled limits approach [38].

2.2.5.2 Simple scaled BE limits

The BE scaled limits approach might be preferable as the relevant confidence limits can be estimated by applying the usual t-statistics. The general form of a scaled BE limit is expressed by Equation 6:

$$(6)$$

$$\text{Upper, Lower BE limit} = \exp\left(\pm k \cdot \sigma_W \right)$$

where k refers to the scaling factor quoted in Equation 4 above. Several rationales have been proposed for setting the proportionality factor of the scaled BE limits [16,35,36].

From Equation 6, it becomes obvious that these limits are continuously increasing with σ_W^2 (**Figure 1C**) and large differences between the means can be accepted with substantial probability. In order to overcome this demerit, several variants have been proposed such as the application of a mixed scaled criterion, the concomitant use of a constraint on the geometric mean ratio (GMR) values, and the use of leveling-off BE limits [32,34,36,40,41].

2.2.5.3 Mixed scaled procedure

According to the mixed method, the classic unscaled criterion is used when the drugs under comparison do not exhibit high variability. However, when σ_W^2 exceeds a preset variability value (σ_{W0}), SABE approach takes control over the determination of BE [36]. According to this proposition [36], the variability value, σ_{W0}, was set equal to 0.20 which leads the proportionality constant in Equation 6 to get a value of $k = ln(1.25)/\sigma_{W0} = 1.116$ (**Figure 1D**). It is worth-mentioning that the mixed BE model can be converted to a mixed approach of scaled BE limits, using the classic unscaled criterion up to $\sigma_W = 0.20$ and scaled BE limits for $\sigma_W > 0.20$.

2.2.5.4 Scaled procedure with an additional constraint

Scaled methods can lead to BE conclusion even when large differences between the means of the two drug products are present. For this reason, it was proposed to use an additional criterion along with the 90% CI in BE limits [32]. This complementary criterion focuses on the GMR on which a constraint is set on the range 0.80 – 1.25.
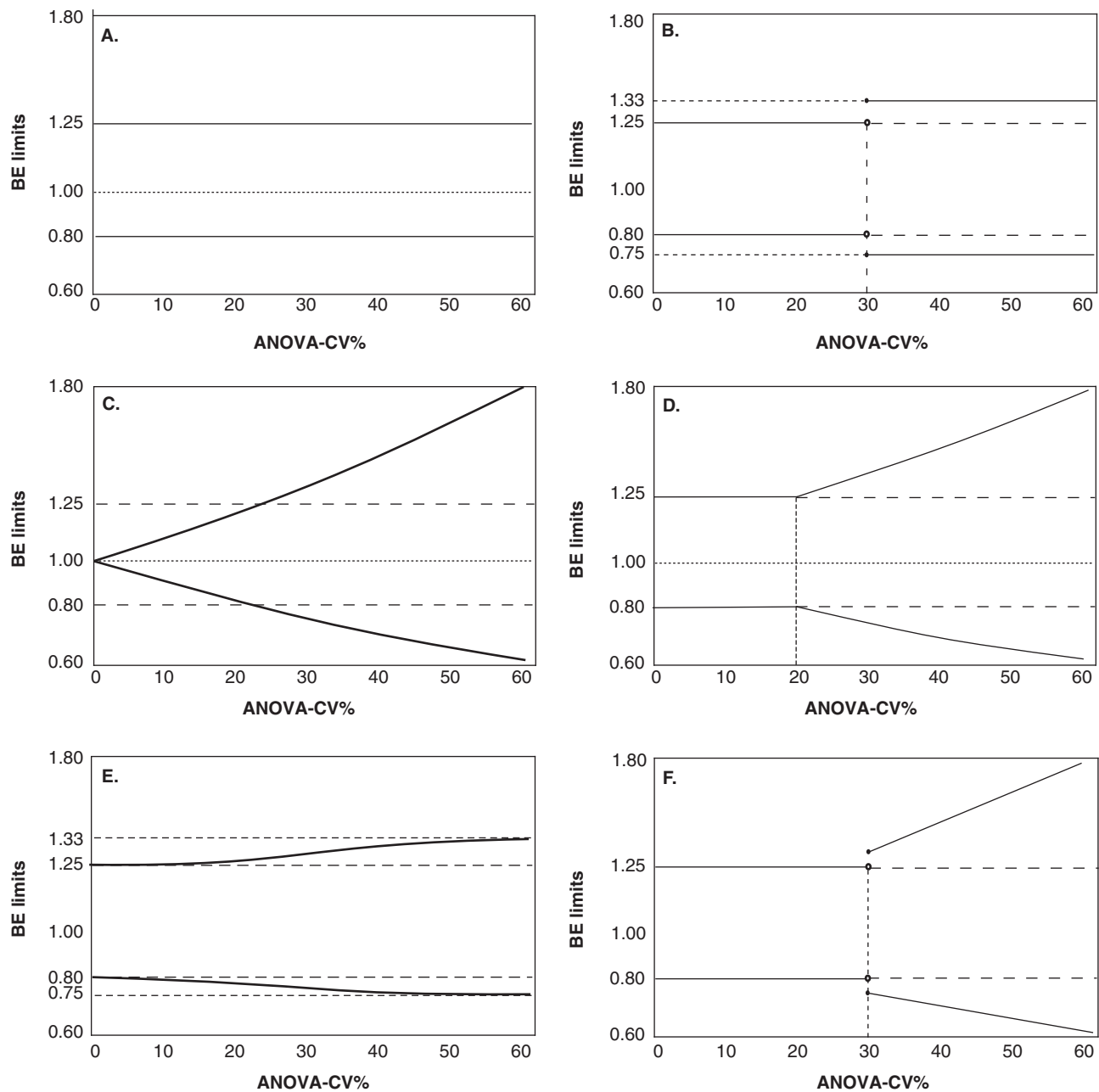
Figure 1. BE limits as a function of within-subject variability (ANOVA-CV%) for: (A) the classic (0.80 – 1.25) limits, (B) extended 0.75 – 1.33 BE limits beyond a specific variability value (30%), (C) scaled BE limits (based on Equation 6) with k = 1, (D) mixed approach (unscaled limits up to 20% and scaled limits for higher variability values using Equation 6 with k = 1.116), (E) leveling-off BE limits based on a sigmoid function [41] and (F) reference-scaled BE limits [42] with a preset variability σ_{reg} = 0.25 and switching variability equal to 30%.

σ_{reg}: Regulatory standardized variation; BE: Bioequivalence; k: Proportionality constant in scaled BE criteria.

2.2.5.5 Leveling-off BE limits

The leveling-off scaled BE limits were introduced as an approach to improve the performance of the scaled BE limits. Leveling-off limits scale with σ_W^2 (as the other scaled limits), but include also a GMR-dependent criterion [34,40]. The latter acts as a constraint on scaled limits and makes them less permissive as the T/R ratio of the study deviates from unity. More recently, a novel leveling-off rationale was proposed [41]. These new BE limits scale with σ_W but only until a maximum plateau value. Hence, the leveling-off BE limits combine the

classic (0.80 – 1.25) and expanded (0.75 – 1.33) BE limits into a single criterion (**Figure 1E**).

2.2.6 Current thinking of regulatory authorities: FDA, EMA and Health Canada

2.2.6.1 The FDA

Recently, a scaled approach was recommended by FDA scientists for the assessment of BE [42]. A replicate design is proposed according to which the R product should be administered twice in each individual, while the T formulation only once; thus, three sequences are possible, TRR, RTR and RRT. For σ_W^2 values of R greater than a pre-set variability cutoff point (30%), a reference-scaled with σ_{WR} average BE approach is suggested (**Figure 1F**). Additionally, a point-estimate constraint is imposed on the GMR of T and R products so that the GMR be limited to the range of 0.80 – 1.25. It was suggested [42] that a value of 0.25 should be assigned to σ_{reg} as the latter demonstrates a good balance between a conservative approach and a practical one. Mathematically, this criterion can be described by Equation 7:

$$\text{Upper, Lower BE limits} = \exp\left(\pm \ln(1.25) \cdot \frac{\sigma_{WR}}{\sigma_{reg}} \right), \tag{7}$$

$$\text{for } \sigma_{WR} \geq 0.30$$

where σ_{reg} refers to the regulatory standardized variation which is set equal to 0.25.

2.2.6.2 The EMA

In case of HVD, the new European Medicines Agency (EMA) guideline recommends the use of a replicate crossover design [12]. According to this design, the R product should be administered twice, thus, allowing the estimation of the σ_W^2 of the R formulation. The acceptance limits of AUC remain unchanged (i.e., 0.80 – 1.25) regardless of the level of variability. However, the estimated variability values for C_{max} can be used to construct scaled BE limits (similar to Equation 6) for C_{max} up to a maximum of 69.84 – 143.19%. The value of the regulatory constant, k, of Equation 6 is now set to 0.760. In any case, the GMR of the study should lie within the conventional acceptance range of 0.80 – 1.25.

A novel issue, in the recent EMA guideline, is the possibility of conducting a two-stage design. According to this statement, an initial group of subjects can be treated and their data analyzed. If BE cannot be demonstrated, an additional group can also be recruited, preserving the overall type I error of the experiment. Eventually, the results from both groups are combined in a final analysis.

2.2.6.3 Health Canada

The Canadian regulatory authorities adopt a different perspective for the assessment of BE. In case of AUC, the typical criteria (Equation 1) for the inclusion of the 90% CI in the 0.80 – 1.25 acceptance interval are recommended. However,

for C_{max}, only its point estimate is used and not the 90% CI [43]. It is worth mentioning that this is a general requirement applied to all drugs and not only to HVDs. However, it becomes beneficial for HVD, as variation of C_{max} is usually higher than that of AUC and the 90% CI of C_{max} can be rather wide. In other words, BE is concluded when the T/R ratio of C_{max} falls within the 0.80 – 1.25 limits, instead of its 90% CI.

2.3 Biowaivers

BE studies can be substituted with other type of evidence, such as *in vitro* data, to save time and reduce cost. The term biowaivers refers to these exceptions to the requirement of performing clinical studies [13,44]. The most common type of biowaivers includes the application of biopharmaceutics classification system (BCS) and the biopharmaceutics drug disposition classification system (BDDCS) [12,44-46].

2.3.1 BCS

BCS was proposed as a framework to classify drug substances according to their solubility and permeability properties [45]. According to BCS, drugs are classified into four categories (**Figure 2A**). The class I of BCS comprises drugs with high aqueous solubility and high membrane permeability, while drugs (usually weak acids) classified as class II are those with poor aqueous solubility and high permeability. In class III of BCS are lying drugs with high aqueous solubility and poor membrane permeability. Finally, drugs with poor aqueous solubility and poor membrane permeability are classified into class IV.

The successful application of BCS requires its combination with the dissolution properties of the drug. A drug substance belonging to BCS Class I can claim biowaiver if it dissolves very rapidly (i.e., > 85% dissolution in < 15 min) in media with pH 1.2, 4.5 and 6.8. Similarly, if dissolution occurs just rapidly (namely, > 85% in 30 min), BE studies can be waived if the similarity factor (f_2) value exceeds the value of 50 [47,48]. According to EMA guidelines, waiver of *in vivo* BE studies can be claimed for BCS I and III drugs [12]. The scientific basis for granting biowaiver for class III drugs is ascribed to the fact that these compounds are highly soluble. Hence, if their formulations dissolve rapidly, absorption will be controlled by gastric emptying and drug permeability. Assuming that excipients do not affect absorption process, drug permeability can be considered to be independent from the formulation properties [12]. However, in the US, biowaiver can be granted only for BCS class I drugs [44].

2.3.2 BDDCS

BDDCS, which was initially based on BCS, was proposed to early address issues related to drug disposition and drug interactions in the intestine and liver [46,49,50]. Drugs belonging either to class I or II of BDDCS are eliminated primarily via metabolism, while those classified into the third or fourth class are eliminated

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A.　　　　BCS

Class II	Class I
High permeability	High permeability
Low solubility	High solubility
Class IV	Class III
Low permeability	Low permeability
Low solubility	High solubility

B.　　　　BDDCS

Class II	Class I
High metabolism	High metabolism
Low solubility	High solubility
Class IV	Class III
Low metabolism	Low metabolism
Low solubility	High solubility

Figure 2. A. BCS divides substances into four classes according to their permeability and solubility values. **B**. BDDCS categorizes drugs based on their metabolism and solubility properties.

BCS: Biopharmaceutics classification system; BDDCS: Biopharmaceutics drug disposition classification system.

unchanged by the kidneys or the biliary route (**Figure 2B**). In other words, for drugs already in the market, knowledge of metabolism can lead to predictions of drug permeability without costly and time consuming human permeability studies. In case of new molecular entities, BDDCS provides a road map for the design of preclinical and Phase I clinical studies.

2.4 Metabolites

The role of drug metabolites (M) in the determination of BE represents another unresolved issue in the field of BE [51-55]. Usually, BE studies are carried out focusing only on the measurement of the parent drug (P). The latter relies on the fact that the concentration–time profile of P is more sensitive to detect differences in formulation performance than M [54,55]. In addition, when the administered drug is not metabolized or is the only active substance, the regulatory authorities recommend the use of the parent drug for BE assessment [12,13]. However, there are situations where metabolite data could exert a significant role in BE assessment. Such cases include the conditions where the parent drug levels in biological fluids are low to allow an accurate analytical measurement, P is an inactive pro-drug or is unstable in the biological fluids, and when M contributes significantly to the net activity with a nonlinear underlying PK system [11,13,54,56].

Several approaches have appeared in literature to investigate the role of metabolites in BE assessment [52,57-64]. Some of them are based on actual BE studies, while other methodologies rely on the generation of simulated data. A recently published study highlighted the fact that the analyte of choice would be the one which carries the information of BE with higher sensitivity in detecting differences between the T and R formulations and lower variability of this response [65]. According to this analysis, both P and M data share the same ability to declare BE when AUC is used as a BE measure. In case of C_{max}, metabolite data exhibit better performance when the two drugs are truly bioequivalent or they only differ in their extent of absorption.

2.5 Biosimilars

Biotechnology-derived macromolecules represent a new class of drugs which include therapeutic compounds such as peptides/proteins, monoclonal antibodies (mAbs), antisense oligonucleotides and cytokines. This evolution undoubtedly enhanced the treatment efficacy of serious diseases, but at the same time several issues are raised regarding the quality of these new types of drugs and the fact that the patents of several of these biologicals have already expired or are about to expire. Therefore, it is a worldwide need to define the regulatory framework for the evaluation of potential similar biological drugs.

Using a methodology similar to that of low molecular drugs, namely, proving essential similarity is disputed by the regulatory authorities both in the US and Europe. It is currently accepted that that it is not possible for two different manufacturers to produce two identical biological products. A case-by-case evaluation approach should be applied, even though some general principles and recommendations may also apply. According to the EMA, a centralized procedure is obligatory. Special emphasis should be placed on the quality, safety and efficacy of the final product which is required to be compared with the R product authorized in the EU [66,67]. A close clinical monitoring of the biopharmaceutical product is necessary even during the post-approval period. The European authorities have already issued specific annexes for several product classes for recombinant erythropoietins, insulin and mAbs [68-70].

3. Conclusion

BE refers to the absence of a significant difference in the rate and extent to which the active moiety of drug products becomes available at the site of action. Classically, determination of BE relies on average BE which is usually based on the use of two-period, two-treatment 2×2 crossover studies. However, several problems have been arisen during the last years. The aim of this review is to summarize the basic

concepts of BE assessment and to offer an insight into the novel approaches of BE assessment regarding HVDs, biowaivers, metabolites and biosimilars.

4. Expert opinion

The ultimate goal of BE is to assess the expected *in vivo* equivalence of two drug preparations of the same active moiety. Classically, BE assessment relies on the concept of average BE where in most of the cases a *2 × 2* crossover study design is applied. During the past years, scientists worldwide recognized the demerits of this procedure and several alternatives have been proposed and adopted by regulatory authorities. However, these methods still suffer from some limitations.

Recently, the FDA working group suggested that for HVDs a reference SABE approach along with a secondary constraint criterion on GMR should be applied for the determination of BE [42]. However, several questions may arise from this procedure such as what is the definition of a highly variable criterion and how drugs exhibiting borderline variability should be treated. Besides, this approach suggests the use of a regulatory standardized variation (σ_{reg} = 0.25) lower than the switching coefficient of variation (30%) which makes the BE limits discontinuous at the switching variation value [71]. If these two values are set at the same level (e.g., 30%) then the difficulty of discontinuous probabilities would be avoided. Besides, studies focusing on human exposure revealed that the ability of the reference-scaled approach to lower the producer risk at GMR = 1 was totally counterbalanced by the rise of consumer risk at high GMR values [72].

Another BE issue still unresolved is that of generic antiepileptic drugs (AEDs). Several concerns have appeared in the scientific community regarding the switchability of the AEDs, namely, to switch a patient from one product to another formulation of the same active substance. For AEDs, the risk of shifting between a brand name and a generic product is more serious due to the nature of epilepsy and the possible problems which may arise in the social life of the patient (e.g., job difficulties, loss of driving privilege). Thus, these drugs require a special BE framework to avoid therapeutic failures [73].

In addition, phenotyping and/or genotyping of subjects are issues that require more attention in BE assessment. Both the 2001 and the 2010 EMA guidelines quote that phenotyping/ genotyping of subjects could be supported for safety or PK reasons [11,12]. Besides, these methods could also be advantageous in case of highly-variable drugs, as these approaches can decrease variability not related to the pharmaceutical dosage form.

Future expectations in the field of BE offer a significant role to biowaivers which allow the use of evidence other than *in vivo* testing. Several types of biowaivers are currently in effect such as the cases of oral solutions and intravenously administered products. A more active role of biowaivers in BE assessment is expected in the near future. The latter may include the further use of nonlinear *in vitro–in vivo* correlations [74], clarification of the ambiguities regarding the biowaivers for class III (or even for class II) of BCS [75] and complete elucidation of the role of transporters' effects in pharmacokinetics in the framework of BDDCS.

In this vein, the further use of *in silico* methods will allow predictions for drug absorption, distribution, metabolism and excretion profile relying only on *in vitro* data and the knowledge of the underlying system. These kinds of computational methods are continuously enriched with features such as genetic polymorphism information, physiological and pathological factors, and transporter effects which will lead to better predictions of the *in vivo* performance and allow population-based predictions.

Another challenge for the future is the equivalence assessment of biotechnology-derived drugs and the BE of the special types of new drug delivery systems (e.g., immuneliposomes, nanopeptides, dendrimers), which now are starting to appear in the market. This rapidly changing field requires the development of new assessment strategies to overcome certain requirements such as immunogenicity, long-term toxicity and complete characterization of the process.

Declaration of interest

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