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Synergic development of pharmacokinetics and bioanalytical methods as support of pharmaceutical research

M Marzo, R Ciccarelli, P Di Iorio, P Giuliani, F Caciagli and A Marzo

Abstract

The development of pharmacokinetics led this science to achieve a relevant role in the investigation of new chemical entities for therapeutic application, and has allowed a series of new useful realizations of out of patent drugs like prolonged release and delayed release formulations, therapeutic delivery system (TDS) for drugs to be active in systemic circulation avoiding the first pass effect, orodispersible and effervescent formulations, intramuscular and subcutaneous depot formulations acting over a long period, oral inhalatory systems, and drug association at fixed dose. The above applications had pharmacokinetics as protagonist and have required the support from bioanalytical methods to assay drug concentrations, even in $pg \cdot mL^{-1}$ of plasma, that really have paralleled the synergic development of pharmacokinetics.

The complexity of the above realizations required specific guidelines from the regulatory authorities, mainly the US FDA and EU EMA, which have normalized and, in most cases, simplified the above applications admitting some waivers of *in vivo* bioequivalence.

However, this review highlights some critical points, not yet focused on by operating guidelines, which need to be clarified by regulatory authorities. One of the most relevant issues is about the planning and conducting bioavailability and bioequivalence trials with endogenous substances, that possess own homeostatic equilibria with fluctuations, in some cases with specific rhythms, like melatonin and female sex hormones. The baseline subtraction required by guidelines to define the net contribute to the exogenous absorbed drug in most cases is a non-solvable problem.

Keywords

analytical bioassay methods, bioavailability, bioequivalence, endogenous substances, pharmacokinetics

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Introduction

The name pharmacokinetics was coined by Dost in 1953.[1] This science initially developed slowly, and then quickly, paralleling the development of analytical bioassay methods that now are able to quantify plasma concentration of drugs possessing high distribution volume (V_d), reaching low limit of quantification (LLOQ) with values ranging from $pg \cdot mL^{-1}$ to $ng \cdot mL^{-1}$. This is now achievable with chromatographic methods, mainly with the tandem mass spectrometry (LC-MS-MS) that assures the highest specificity, sensitivity, and short-lasting analytic runs.[2]

The guidelines edited by the US FDA and EU EMA have progressively directed and clarified several aspects in planning and performing pharmacokinetic (PK), bioavailability (BA), and bioequivalence (BE) trials. Some other questionable aspects classified in literature as 'open questions on BE' still exist and need a definite clarification

Department of Medical, Oral e Biotechnological Sciences, "G. d'Annunzio" University of Chieti-Pescara, Via dei Vestini 31, I-66013, Chieti, Italy

Corresponding author:
Antonio Marzo, Department of Medical, Oral e Biotechnological Sciences, "G. d'Annunzio", University of Chieti-Pescara, Via dei Vestini 31, 66013, Chieti, Italy.
Email: antoniop.marzo@libero.it

by regulatory authorities.[3–5] The main open question not yet clarified is that related to the PKs and BE of endogenous substances that in most cases do not present well evident peak shapes after oral administration and thus do not allow the baseline subtraction required by guidelines.[6]

Development steps in pharmacokinetics as substantiated by analytic improvement

Radiotracing approach

This was the first pharmacokinetic approach and was characterized by the administration of drugs labeled with ^{14}C or ^{3}H. PKs were studied following the total radioactivity with the liquid scintillation technique. This technique is not specific as evaluates together parent drug and metabolite(s). The first attempt, that should be considered pioneering, was that of Okita et al. who developed *Digitalis purpurea* into an airtight growing chamber in the presence of $^{14}CO_2$. After 2 years, the above investigators extracted and purified ^{14}C-digitoxin from the leaves and administered this cardiac glycoside to dogs and humans, thus producing the first PK data on this drug.[7–9] The further development was to randomly label drugs with ^{3}H with a $H/^{3}H$ exchanging chromatographic process. Doherty et al. produced the first data on ^{3}H-digoxin, another cardiac glycoside largely used in that period to treat congestive heart failure and other circulation disorders.[10]

The radiotracing technique was largely used to achieve PK data on several drugs,[11,12] but was quickly neglected when more specific techniques were available. This technique, however, is still used in the first development steps of drugs to evaluate the ADME (absorption, distribution, metabolism, excretion), namely the whole amount of the given drug that is excreted via urine, via feces and is still present in the body. This trial is carried out in both animals and healthy volunteers.

Radioimmunoassay (RIA) approach

This method had its relevant promoter Lindenbaum who, using the RIA of digoxin just set up by Smith et al.,[13] bioassayed plasma concentrations of this cardiac glycoside after administration of four different brands to four subjects following the crossover design. Very relevant differences in digoxin profiles were observed comparing the four brands administered.[14] This led the US FDA to prescribe a dissolution test for digoxin tablets that assured in further lots an acceptable uniformity. Lindenbaum used the term 'biological availability' that was quickly contracted in bioavailability. The experiment of Lindenbaum introduced not only the above term in the literature, but in particular the concept of bioavailability in the mind of scientific operators, that means the possibility to obtain plasma concentrations specific of a defined pharmaceutical formulation.

The RIA was applied only to a restricted number of drugs. It represented an improvement if compared to the previous radiotracing approach, but emerging chromatographic techniques quickly prevailed. Now RIA is still used to quantify some endogenous substances for PKs, BA, and BE.

Chromatographic approach

In comparison to radiotracing and RIA, chromatographic techniques allow a better specificity as the column involved can separate the peaks of the various analytic components, e.g. parent drug and metabolites or any interfering substance.

The first chromatographic technique used in PKs was gas chromatography (GC), which however needs the analyte to be heated in order to become volatile. All functional groups like hydroxyl (-OH), carboxyl (-COOH), and amino ($-NH_2$, -NH-) must be derivatized to give esters, ethers, and amides that can become volatile. Detectors like flame ionization (FID), thermoionic specific (TSD), electron capture (ECD), and mass fragmentography (MS) allow a quantitative evaluation of the analyte eluted from the column.[15,16] High performance liquid chromatography (HPLC) and capillary electrophoresis (CE) do not need any heating and use UV-Vis, or electrochemical or fluorimetric detectors. With both GC and HPLC the analytical run lasts several minutes, e.g. until 18 min, and the LLOQ that can be obtained is in the range of 20–100 $ng \cdot mL^{-1}$ (Tables 1 and 2). CE did not meet analytical development in bioassay techniques as, in spite of the very high sensitivity, it requires a very low volume of sample injected that vanishes its appeal of sensitivity.

Both HPLC and GC techniques were surpassed by LC-MS-MS and are still used for some applications, mainly in pharmaceutics.

Table 1. Comparison of main analytical features between HPLC and LC-MS-MS bioassay of nimesulide and its active metabolite 4-hydroxy-nimesulide.

	HPLC	LC-MS-MS
Drugs	Nimesulide and 4-hydroxy-nimesulide (4-OH-nimesulide)	
Detection	UV 330 nm	API 365 triple quadrupole mass spectrometry with turbo ion spray interface
Dynamic range; LOQ	100–8000 ng·mL^{-1}; LOQ 100 ng·mL^{-1}, with both analytes	0.5–80 ng·mL^{-1}; LOQ 0.5 ng·mL^{-1}, with both analytes
Retention times	Nimesulide 11.0 min, 4-OH-nimesulide 6.1 min, I.S. 14.2 min	Nimesulide 2.42 min, 4-OH-nimesulide 2.18 min, I.S. 2.66 min
Duration of the run	18 min	5.5 min
Sample size of the run	50	150
Time required for 1000 analyses by one operator	20 days	7 days

Table 2. Comparison of main analytical features between GC and LC-MS-MS bioassay of isosorbide-5-mononitrate.

	GC	LC-MS-MS
Drug	Isosorbide-5-mononitrate	
Detection	ECD ^{63}Ni	API 365 triple quadrupole mass spectrometry with turbo ion spray interface
Dynamic range; LOQ	20–700 ng·mL^{-1}; LOQ 20 ng·mL^{-1}	5–80 ng·mL^{-1}; LOQ 5 ng·mL^{-1}
Retention times	Isosorbide-5-mononitrate 8.9 min, I.S. 6.3 min	Isosorbide-5-mononitrate 3.5 min, I.S. 4.2 min
Duration of the run	15 min	5.5 min
Sample size of the run	30	150
Time required for 1000 analyses by one operator	days	7 days

Chromatographic techniques are still now preferentially useful with chiral columns when enantiospecific separation of enantiomers is requested, this both in pharmaceutics and in pharmacokinetics.[17]

Tandem mass spectrometry

The triple quadrupole analytical technique, currently called tandem mass spectrometry, in comparison to the previous analytical techniques, possesses the following advantages:

- the highest specificity
- a high sensitivity, in the range from pg·mL^{-1} to ng·mL^{-1}
- the shortest run time, namely about 4–6 min

The most useful application of triple quadrupole is as detector of liquid chromatography (LC-MS-MS). This technique has allowed to bioassay a number of drugs in biological fluids, including drugs possessing high distribution volume that require high sensitivity to appreciate plasma concentrations of pg·mL^{-1}.[18–25]). A less frequent application of mass spectrometry is as a detector of gas chromatographic eluate.[26–28]

Tables 1 and 2 show compared conditions to bioassay in human plasma nimesulide and its 4-hydroxy metabolite, and isosorbide-5-mononitrate with chromatographic techniques (HPLC and GC) and tandem mass spectrometry.[2] The advantage of tandem mass spectrometry is evident from the run lasting that is 3–5 times shorter, from the sensitivity that expressed from LLOQ is 2–200 times better and from the time requested to bioassay 1000 analyses, that was 3–4 times shorter.

The above characteristics promoted this technique as the most used in pharmacokinetics, bioavailability, and bioequivalence.[2]

Figure 1 shows the development of the analytical techniques to bioassay drugs in biological fluids in the period 1977–2014. Data were drawn from a number of papers in the range of 41–57 per year published in 1977, 1985, 2000, and 2014 in

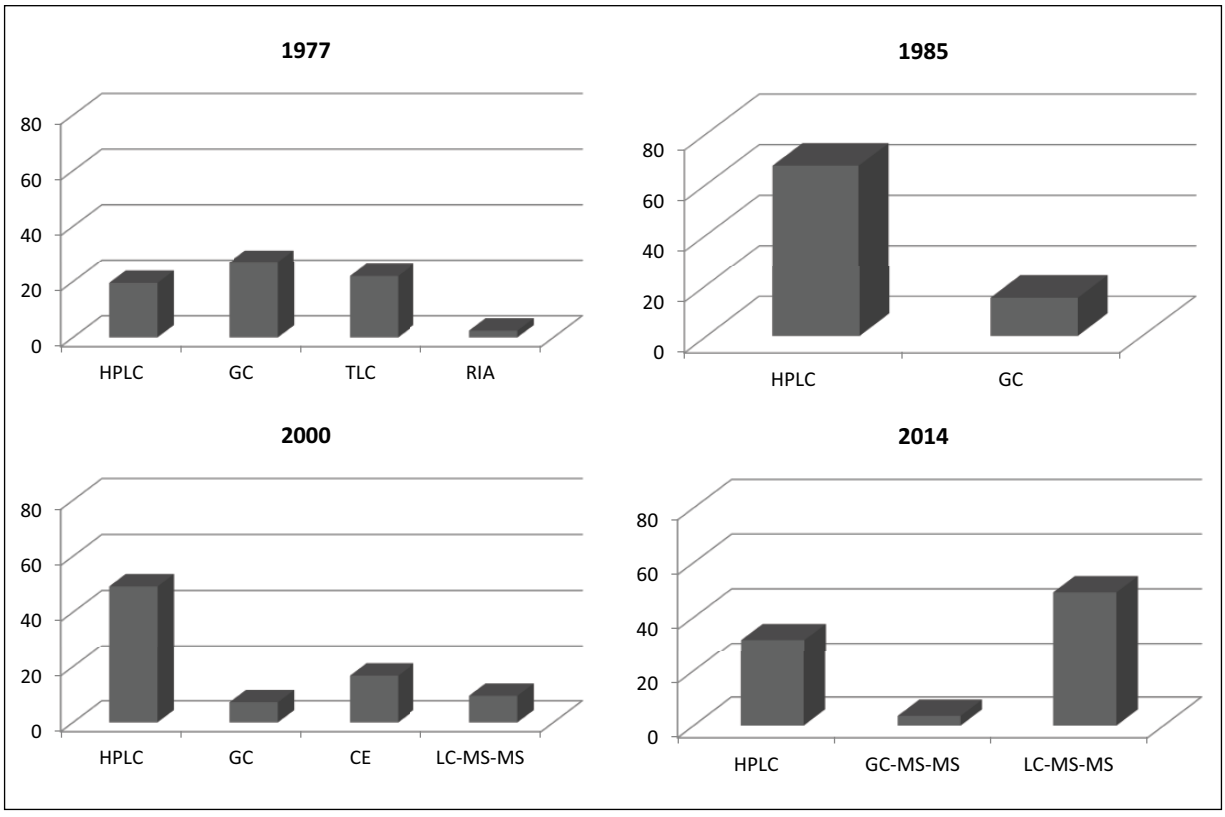

Figure 1. Frequency in the use of analytical techniques for bioassay, as published in the *Journal of Chromatography, Biomedical Applications* in 4 different years.

the *Journal of Chromatography, Biomedical Applications*.

EU guidelines on bioavailability and bioequivalence

The first EU guideline on BA and BE was edited in 1991.[29] Ten years later EMA emitted a further guideline on BA and BE.[30] A deeply revised guideline was edited by EMA in 2010.[31] The last guideline was focused only on bioequivalence of immediate release formulations. The guideline on modified release formulations and transdermal dosage forms was edited by EMA in 1996[32] and updated in draft in 2013.[33] The above guidelines have focused on most specific problems that occur in BA and mainly in BE trials.

However, some critical aspects now still need specific suggestions in order to plan and carry out some specific kind of trials. The main problems are definitely clarified and those not well-defined in the last guideline[31] are discussed in the sections below.

Problems focused on by guidelines

Titer difference between test and reference

In bioequivalence trials, the titer of test and reference must differ from each other by not more than 5%. In the case of a higher difference, BE on C_{max} and AUC must be checked on dose normalized parameters. Previous EMA guidelines did not allow the above dose-normalization. This possibility can be very useful in the case of drugs that are acceptable by pharmaceutics in a titer range larger than 95.0–105.0%.[5]

Carryover from the pre-dose to the second study period

In crossover trials the pre-dose baseline of the second study period in a given volunteer could contain a measurable drug concentration > LLOQ. If this value is <5% of C_{max}, the volunteer can be statistically processed. If the baseline is >5% of C_{max}, the given volunteer must be excluded from the statistical analysis.[31] This statement is in line with US FDA guidelines,[34]

Table 3. Enlarged limit of 90% CI of C_{max} for different values of CV %.

Intrasubject CV %	Enlarged limit of 90% CI of C_{max}
30	0.8000–1.2500
35	0.7723–1.2948
40	0.7462–1.3402
45	0.7215–1.3859
⩾50	0.6984–1.4319

but was not considered in previous EU guidelines. The greater sensitivity achievable with LC-MS-MS has increased the frequency of the presence of the above carryover that can be avoided prolonging the wash out separating the two study periods.[35]

Enlargement of the 90% confidence interval for C_{max}

The first EU guideline (EMA, 1991) allowed, in the case of C_{max}, the 90% confidence interval (CI) for assessing BE to be enlarged from the stipulated 0.80–1.25 interval without, however, giving any limit. From publications the expanded limit was set to 0.70–1.43.[36] The EU guideline edited on 2001[30] indicated how 90% CI of C_{max} could be enlarged in the case of high variability as 0.75–1.33. The last EU guideline[31] allowed 90% CI of C_{max} to be enlarged only in the case of intrasubject coefficient of variation (CV) ⩾30% and if the replicate design was adopted and only for oral immediate release formulations as reported in Table 3.

US FDA guideline on bioequivalence does not allow any enlargement of 90% CI of C_{max} from the statistics 0.80–1.25 range.[34]

Drugs characterized as narrow therapeutic index (NTIDs) with C_{max} of particular importance for safety, efficacy, or drug level monitoring must produce 0.90–1.11 as the acceptance interval for C_{max} to be assessed as bioequivalent.[31]

Parent drug or active metabolite(s)

In the case of parent drug and active metabolite, the last EMA guideline[31] has clarified to bioassay and assess BE only on parent drug. However, in some cases the parent drug disappears quickly so that it is not possible to follow its plasma concentration, whereas active metabolite produces a well evident shape of plasma concentration. Some parent drugs largely biotransformed in active metabolite are reported in Table 4.

In the above and similar cases, the guideline accepts the assessment of bioequivalence on metabolite, even if it is not active, like in the case of betahistine.

Waivers for in vivo *bioequivalence*

EU and US guidelines accept in some specific cases the waiver of *in vivo* bioequivalence trial. Water solutions are exempt. With several doses of a given drug in most cases their approval can be authorized on the basis of a BE trial on only one dose, usually the highest, in other cases the lowest. Also intravenous (IV) injectable solutions are exempt from BE. However, the most interesting case is that arose from the Biopharmaceutical Classification System (BCS) published by Amidon et al.[37] and focused on by guidelines.[31,38] Amidon classified drugs into four groups according to their solubility and permeability (Table 5).

US FDA and EU EMA guidelines[31,38] exempt from *in vivo* BE drugs classified in groups 1 and 3 of BCS, if they however demonstrate to possess the requested solubility: dissolution >85% within 15 min or >85% within 30 min in the pH range of 1–6.8. The waiver is restricted to oral immediate release formulations and drugs not classified as narrow therapeutic index.

The number of drugs targeted as laying in groups 1 or 3 of the BCS increased in the last years and are listed in Table 6, in which the drugs are grouped in class 1 and class 3 of BCS and are susceptible to a BE waiver.[39–41] The enlargement of the number of drugs classified in the two above classes has reduced the need of BE trials. Their approval is supported by studies *in vitro* on the solubility and the dissolution rate at the requested series of pH.

Problems that are still now neglected by guidelines

Multiple peak phenomenon

Some drugs are absorbed producing two or more peaks of plasma concentration.

Typical is the two-peak phenomenon of drugs that meet the enterohepatic circulation, that produce a first peak within 2 h, and a second peak within 6–12 h after administration. This occurs *inter alia* with piroxicam,[4,42] glibenclamide,[43,44] ursodeoxycholic acid,[45] mycophenolic acid, and its parent drug mycophenolate mofetil.[46]

Table 4. Metabolite to parent drug AUC ratio of some largely biotransformed parent drugs.

Active metabolite	Parent drug	Metabolite to parent drug AUC ratio
Enalaprilat	Enalapril	2.5[75]
Zofenoprilat	Zofenopril	6.7[75]
Hydroxyflutamide	Flutamide	60[76]
Hydroxypurinol	Allopurinol	44[77]
Acid metabolite of terfenadine	Terfenadine	⩾100[78]
Monohydroxycarbamazepine	Oxcarbazepine	⩾100[79]
Mycophenolic acid	Mycophenolatemofetil	⩾25[52,80]
Desmethylclozapine N-oxideclozapine and other metabolites	Clozapine	~70% metabolized[81]

Table 5. Four drug classes according to solubility and permeability, by Amidon et al.[37]

Drug class	Solubility	Permeability
1	High	High
2	Low	High
3	High	Low
4	Low	Low

More complex and less predictable are other cases when the second peak or a series of peaks appears quicker, namely within 3–4 h after dosing. Among these cases what happens with diclofenac would be remembered. According to Bettini et al.[47] the second peak of diclofenac found by various authors[2,48–50] is the result of a hydratation process of the drug to tetrahydrodiclofenac, less soluble, occurring in the gut. A formulation of diclofenac able to be absorbed very fast proved to produce only one peak, namely the first one, of relevant entity.[51]

In these cases Marzo and Reiner[3] suggested assessing bioequivalence only on the values of 90% CI of AUC, whereas C_{max} should be managed checking individual values to lay in the range expected from previous literature for the given dose, thus excluding any problem of unexpected activity or tolerability. This suggestion was not considered by EMA neither in the guideline[31] nor in the Questions and Answers.[52]

Ethics problems on bioequivalence

Some drugs can produce safety problems if administered in bioequivalence trials on healthy volunteers. Excluding the case of cytostatic agents that could not be given to healthy volunteers, some other cases should be considered, as follows. Carbamazepine in steady state that requires 30 days to be achievable;[53] cyclosporine that can affect renal clearance;[54] flutamide that can cause gynecomastia in men;[55] morphine for several adverse events;[56] warfarin that requires a long period of treatment to achieve steady state;[57] and clozapine for relevant side effects.[58,59]

In the above cases and, of course, in other similar situations the administration in healthy volunteers, mainly in trials of repeated dose regimen to reach a steady state, should be carefully considered and possibly avoided.[4] Indeed, operating guidelines require steady state to assess BE of extended-release oral formulations, for transdermal delivery systems and for immediate release formulations of drugs that are cleared from the body with long half-lives.[31–33]

Endogenous substances and their baseline

PKs, BA, and BE of endogenous substances in most cases are faced with very complex problems not considered by EU operating guideline. The difficulties in managing trials with these substances are the following:

(a) the presence of a baseline, that can fluctuate around an average level, and can have specific rhythms, as in the case with melatonin, cortisol, and female sexual hormones;[6]
(b) the request from the EU EMA to subtract baseline, that in the above situation (a) often presents relevant difficulties, mainly in the presence of endogenous rhythms;
(c) the homeostatic equilibria of the body that operate in order to avoid excessively high or low concentrations of the endogenous substances through strictly controlled mechanisms;
(d) the dilution effect of the low amount entering the systemic circulation with that more relevant contained in the body;

Table 6. List of drugs classified in classes 1 and 3 of BCS.

Class 1	Class 3
Acetylsalicylic acid	Abacavir
Allopurinol	acyclovir
Amiloride HCl	Alendronic acid
Amitriptyline HCl	Amodiaquine
Amlodipine	Anastrazole
Amoxicillin	Atenolol
Ascorbic acid	Benznidazole
Bisoprolol	Biperiden HCl
Calcium folinate	Captopril
Cetirizine	Carbidopa
Chloroquine sulfate	Cefaclor
Citalopram/escitalopram	Chlorambucil
Clindamycin	Chloramphenicol
Cyclophosphamide	Chlorphenamine hydrogen maleate
Diazepam	Chlorpromazine HCl
Diethylcarbamazine dihydrogen citrate	Cimetidine
Digoxin	Ciprofloxacin HCl
Donepezil	Clavulanic acid
Doxazosin	Clomifene citrate
Doxycycline HCl	Clomipramine HCl
Fluconazole	Cloxacillin sodium salt
Lamivudine	Codeine phosphate
Levodopa	Colchicine
Levofloxacin	Cycloserine
Levonorgestrel	Didanosine
Lithium carbamate	Enalapril
Loratadine	Ergocalciferol
DL-methionine	Ergotamine tartrate
Metronidazole	Ethambutol HCl
Mirtazapine	Ethinylestradiol
Nicotinamide	Ethionamide
Norethisterone	Ethosuximide
Ofloxacin	Ferrous salt
Ondaserton	Flucytosine
Paracetamol	Gabapentin
Phenobarbital	Glyceryl trinitrate
Phenoxymethyl penicillin potassium salt	Hydralazine HCl
Potassium iodide	Hydrochlorothiazide
Pravastatin	Isoniazid
Prednisolone	Isosorbide dinitrate
Primaquine diphosphate	Letrozole
Proguanil HCl	Levamisole HCl
Promethazine HCl	Levetiracetam
Propranolol HCl	Levothyroxine sodium salt
Propylthiouracil	Lisinpril
Pyridoxine HCl	Losartan
Quinapril	Medroxyprogesterone acetate
Quinine sulfate	Metformin HCl
Ramipril	Methotrexate sodium salt
Riboflavin	Methyldopa
Salbutamol sulfate	Metoclopramide HCl
Sertraline	Morphine sulfate
Sildenafil	Neostigmine bromide
Stavudine	Nifurtimox
Tamoxifen citrate	Penicillamine
Terbinafin	Pentamine
Theophylline	Procarbazine HCl
Tramadol	Pyrazinamide
Valproic acid	Pyridostigmine bromide
Venlafaxine	Quinidine sulfate
Warfarin sodium salt	Ranitidine HCl
Zidovudine	Risedronic acid
Zolpidem	Sulfadoxine
	Terazosin
	Thiamine HCl
	Topiramate
	Zinc sulfate

(e) the multicomponent reversible metabolism, common with endogenous substances;[60]
(f) the specific body storages, e.g. bones, fats, red blood cells;
(g) the renal threshold that is a mechanism able to assure constant systemic concentrations of several substances, as for instance L-carnitine, most ions, and some amino acids.[6,60]

The consequence of the above homeostatic mechanisms is that the absorption of endogenous substances administered exogenously in most cases does not produce a well-defined shape of plasma concentration.

In these cases, even when a peak shape is obtained, a serious problem of increased variability arises about the regulatory request to subtract baseline, as follows in the simulation reported as an example in the Scheme 1.

AUC (total value measured)	100 $ng \cdot ml^{-1} \cdot h \pm 20$	CV = 20 %
AUC baseline	80 $ng \cdot ml^{-1} \cdot h \pm 8$	CV = 10 %
AUC net value (total – baseline)	20 $ng \cdot ml^{-1} \cdot h \pm 20$	CV = 100 %

pool size without baseline subtraction = $392 \cdot CV^2 = 392 \cdot 0.2^2 = 392 \cdot 0.04 = 16$ subjects

pool size after baseline subtraction = $392 \cdot CV^2 = 392 \cdot 1^2 = 392 \cdot 1 = 392$ subjects

Scheme 1. Pool size evaluation of an endogenous substance without and after baseline subtraction.[61]

A simulation case was described for cholecalciferol that, after an oral administration of 100,000 IU (2.50 mg), reaches the peak at 7 days whereas the baseline is restored at 100 days.[62,63]

The above considerations lead to conclude that the baseline subtraction should be avoided in most cases.

With drugs excreted via urine, like L-carnitine, most ions, and some amino acids, the bioavailability evaluation from cumulative urinary excretion is preferable to the profile of plasma concentrations.[64,65] With endogenous substances biotransformed the solution is more complex. In certain cases the subtraction can be avoided administering high doses of the drugs, providing a peak shape well evident. In other cases, a repeated dose regimen for a relatively long period could be sufficient to consider also baseline as a product of the exogenous administration. Another solution is to approach the Test *Vs.* Reference comparison with a phase III trial on target population. This approach was followed by Cerutti et al.[66] in assessing BE of levothyroxine.

Specific cases focused on by regulatory authorities are those of potassium, levothyroxine, and omega 3 derivatives. In the case of potassium, the US FDA guidance suggests assessing bioequivalence on the basis of urinary excretion of this ion and suggests neglecting the plasma concentration of this ion as it is not an expression of bioavailability.[67] In the case of levothyroxine the specific guideline edited by the US FDA suggests administering a high oral dose that produces a relevant peak shape and to avoid baseline subtraction.[68] The EU EMA on the Questions and Answers document has focused on the case of omega 3 derivates eicosapentaenoic acid and docosahexaenoic acid to assess bioequivalence according an exemption justified by the fact that the soft gelatin capsule contains only the above two drugs without any excipient.[52]

Preanalytical preparation of samples for chromato-graphic bioassay

All chromatographic techniques, namely HPLC, LC-MS-MS, and GC, require a previous extraction of the analytical compounds from the matrix that can be achieved with liquid-liquid and solid-liquid extraction, and the addition of internal standard (IS).[15] In the case of LC-MS-MS the best IS should be the deuterated analyte, which possesses identical chemical characteristics of the true analyte, but can be identified and detected by the multiquadrupole analytical system.

The analyst must pay attention to some relevant aspects, as follows:

(a) the plasma-red cell distribution of the analyte. In the case of asymmetric distribution, the procedure must be focused on, e.g. bioassay the analyte on whole blood;
(b) if reversible metabolism is operating, both parent drug and metabolite must be assayed; alternatively the two or more compounds must be chemically transformed in one of them;
(c) in the presence of hydrolytic metabolism active on parent drug, the test tube containing blood before centrifugation must be put in one ice-bath and centrifuged at a planned time, the shorter possible, and then must be frozen in ice-dry and then put in freezer. This is *inter alia* the case of acetylsalicylic acid;
(d) the anticlothing agent must be selected at the validation of analytical procedure and maintained along the further bioassay of samples.

Guidelines on analytical validation

On 1992 Shah et al. published the first suggestions how to manage and validate a bioanalytical method.[69] After a decade the same authors published an updated revision of their previous considerations.[70] Specific guidelines on analytical validation were edited by the US FDA[34] and by the EU.[71,72]

The above guidelines focused on detailed procedures for the pre-study validation that must be extensively described in the Validation Report and for study specific validation that must be documented in the Analytical Report. A specific section is devoted to incurred sample reanalysis, that is considered also in Questions and Answers.[52]

Adjunctive remarks

The following adjunctive remarks should be considered by regulatory agency, which would help the scientific operators in planning their pharmacokinetic research.

Drugs with narrow therapeutic index are excluded from several waivers, as noticed in the present review. However the lack of its univocal definition makes problematic its application.

In certain cases the repeated dose design, required by operating guidelines, can produce tolerability problems that would suggest avoiding this design, adopting only that in single dose. Ethics problems discussed in the present review are typical examples of drugs that should not be administered to healthy volunteers in repeated dose regimen. However, guidelines on bioequivalence do not focus on this aspect.

Another problem that is controversial is whether to use healthy volunteers with PK trial instead of a phase III clinical trial in same applications that do not involve bioavailability, but only local treatment for local activity. According to personal experience, the PK comparison of some test vs. reference, have produced extremely high coefficient of variation that would vanify any bioequivalence assessment. The responsibility of the above very high CV was considered difficult in calibrating the planned dose of test and reference. On the contrary, we have obtained satisfactory results in comparing these topical drugs, test vs. reference, for local and systemic tolerability in healthy volunteers treated for a complete therapeutic cycle. This kind of design in the past was accepted by AIFA in Italy to assess BE for a period of about 3 years. This approach could be considered by the EU at least for certain cases as an alternative to a Phase III trial in assessing bioequivalence of topical drugs for local activity.

Discussion

The updated research of new chemical entities for therapeutic use involves thousands of molecules that must be screened and investigated in order to find one or some interesting among them for new drug application (NDA). This compels scientific operators to design and follow a screening schedule that involves PKs just at the first steps to evaluate the couple of solubility and permeability, in order to classify the molecules in the four classes of BCS of Amidon.[37] The scarcity of new chemical entities in the last two decades has opened an increased space for applied research evolving out of patent drugs addressed to their new applications, as follows. Prolonged release oral formulations that allow the once-a-day regimen, even with drugs possessing a short $t_{1/2}$, like nifedipine, whose half-life is about 2 h. Delayed release formulations of drugs that must act on the colon and if administered orally must be formulated in order to release the active ingredient only after the ileo-cecal valve, in the colon, like mesalazine. Transdermal delivery systems (TDS) for systemic activity that can overcome the first pass effect that metabolizes presystemically a relevant part of the drug, around 99% in the case of nitroglycerin.[73] Female sex hormones, clonidine, and nicotine are also formulated in TDS. Depot formulations of drugs injected i.m. or s.c. allow sustained pharmacological activity that persists over 1 month. Relevant applications are also orodispersible and effervescent formulations of various drugs, oral inhaler systems for treatment of asthma, and new fixed-dose combination products.[74]

The above applications in most cases were reached on research of BE assessment comparing the given drug on test vs. reference formulations according to the ANDA procedures that exempted out of patent drug from clinical trials on target population, considering already well-achieved data on their activity and safety. These applications have as primary protagonist pharmacokinetics that, however, needed the invaluable support of analytical procedures, the LC-MS-MS being the most relevant. All these realizations were normalized and governed by operating guidelines, both the US FDA and EU EMA, which allowed some waivers of *in vivo* bioequivalence in most cases supported by *in vitro* data on solubility, disgregation, and dissolution.

Some open questions still need to be considered and governed by guidelines, the most relevant being the puzzle of endogenous substances that are strictly controlled in the body by homeostatic equilibria, an obstacle in most cases of the evidence of well-defined peak shape after an exogenous administration, and to find net plasma concentrations resulting from the baseline subtraction.

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