DEVELOPING INTEGRATED PHARMACOKINETICS AND BIOSEPARATION METHODS FOR DRUG DISCOVERY

Abstract

The advancement of pharmacokinetics has allowed this science to take the lead in the discovery of novel bioactive molecules for clinical use, resulting in a wide range of off-patent drug formulations such as prolonged and delayed release, therapeutic delivery systems, and so on (TDS). Orally disintegrating and effervescent preparations, intramuscular and subcutaneous depot formulations are used to permit drugs to circulate systemically, eradicating the first pass effect.

Bioanalytical approaches were necessary for measuring drug concentrations, even at the plasma level, in the aforementioned applications that mirrored the development of pharmacokinetics in a synergistic manner. In response to the intricacy of the aforementioned realisations, the US FDA and EU EMA have produced guidelines, and the majority of the aforementioned applications have been streamlined and standardised to include waivers of in vivo bioequivalence.

However, there are a number of points that must be explained by regulators but are not yet addressed in operational standards. Concerning the planning and execution of bioavailability and bioequivalence tests with endogenous compounds, there is an essential issue. Particularly relevant are endogenous chemicals, such as melatonin and female sex hormones, because they have their own homeostatic balance with fluctuations. In most circumstances, quantifying the net contribution of the exogenous drug to the exogenously absorbed drug based on the needed baseline subtraction is not simple.

Keywords: Therapeutic Delivery System, Bioanalytical methods, Endogenous substances, Bioequivalence, Drug Discovery

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I. INTRODUCTION

In 1953, Dost formulated a theory that would later become known as pharmacokinetics. The development of analytical bioassay methods, which enabled the quantification of plasma concentrations of pharmaceuticals with high distribution volumes (Vd) and low limits of quantification (LLOQ) ranging from pg•mL1 to ng•mL1, sped up the progression of this discipline. These methods can be used to measure concentrations of pharmaceuticals in plasma with distribution volumes that range from pg•mL1 to ng•mL1. In recent years, a novel combination of chromatographic technologies known as tandem mass spectrometry (LC-MS-MS) has been invented in order to achieve this goal. This method assures the highest possible level of specificity, sensitivity, and short-term sensitivity. [1]

The US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have published guidelines clarifying a number of aspects of pharmacokinetics, bioavailability, and bioequivalence clinical trials. Regulatory authorities still need to clarify some other questions regarding the BE categorised in literature as 'open questions'. [2] After oral administration, endogenous substances' PKs and BEs are still unclear, so baseline subtraction as required by guidelines cannot be performed. Their peak shapes are often difficult to observe after oral administration because they do not present well-defined peaks.

II. ANALYTICALLY SUBSTANTIATED PHARMACOKINETIC DEVELOPMENT STEPS

Radiotracking strategy: Among the earliest and most widely used approaches to pharmacokinetics, this deal was characterized by the use of radioactive drugs that were labelled ^{14}C or ^{3}H. A liquid scintillation technique was used to study the PKs following the total radioactivity using 14C or 3H as the radioactive element.[3] Since parent drug and metabolite(s) are evaluated together, this technique is not specific. As an example of pioneering research, the first attempt was to develop a growing chamber for Digitalis purpurea in the presence of 14CO2 in a number of airtight environments. The above investigators obtained the first PK data on 14C-digitoxin after two years of analyzing the leaves of this plant and extracted and purified 14C-digitoxin from them, which was administered to dogs and people.[4]

In a later development, after random labeling with 3H was developed, a chromatographic process that exchanges H and 3H was devised to label drugs randomly. As another cardiac glycoside used to treat congestive heart failure and other circulation disorders during that era, 3H-digoxin was also widely used as a treatment for those ailments.[5]

Several drugs have been shown to have poor PK data when measured by radiotracking, but this has not been taken into account when more specific approaches are available. The ADME (absorption, distribution, metabolism, excretion) is still evaluated in early stages of drug development in order to determine how much a drug is excreted by urine or feces, and how much remains in the body after it has been excreted. A healthy volunteer as well as animals are involved in this study. [6]

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An approach based on radioimmunoassay (RIAs): In order to figure out the plasma concentrations of this cardiac glycoside, we used a methodology named a crossover and gave four different brands of cardiac glycoside to the same four patients. We discovered extremely significant changes in the digoxin profiles of the two brands of digoxin when we compared them to four other brands that were administered at the same time. As a direct consequence of the Lindenbaum experiment, the term "bioavailability" was introduced to the scientific community for the very first time, as was the idea of obtaining plasma concentrations of a defined pharmaceutical formulation. To scientists, this signifies the possibility of obtaining particular plasma concentrations for a particular formulation. Additionally, the Lindenbaum experiment was responsible for introducing the concept of obtaining plasma concentrations of a defined pharmaceutical formulation. [7] It is important to highlight that the RIA was only introduced for a relatively small number of different medications. It was a significant advancement in terms of efficiency in comparison to the radiotracing method that had been used in the past; nevertheless, new chromatographic methods emerged almost immediately and swiftly overtook it. For a variety of reasons, it is still necessary to use RIA in order to quantify some of the endogenous chemicals that are found in PKs, BAs, and BEs. [8]

An approach based on chromatography: Since a chromatographic column can separate the peaks of distinct analytic components, like the parent drug and metabolites, the chromatographic approach offers a higher level of specificity compared to radiotracking and RIA. Gas chromatography was initially used for PK analyte analysis (GC). [9] Even though GC has been around for quite some time, the process of heating analytes to make them volatile has been around for quite some time as well. Esters, ethers, and amides are volatile compounds that come from the derivation of functional groups such as hydroxyls (-OH), carboxyls (-COOH), and aminos (-NH2). [10] It is possible to quantitatively analyse the analyte eluted from a column with detection techniques like flame ionisation (FID), thermionic specific detection (TSD), electron capture detection (ECD), and mass fragmentation detection (MS). [11]

Unlike high-performance liquid chromatography and capillary electrophoresis, these techniques do not involve heating, instead relying on UV detectors, electrochemical detectors, or fluorimeters. Both gas chromatography (GC) and high-performance liquid chromatography (HPLC) take a while to complete an analysis (20–100 ng•mL1 for GC) and hence have high detection limits (DDLs) (Tables 1 and 2) [12]. Due to the low sample volumes required for CE, it is insufficient for the analytical advances in bioassay techniques. Although HPLC and GC have been replaced by LC-MS-MS, they are still used for a number of different purposes, particularly in the pharmaceutical industry. When enantiospecific separation is relevant to both pharmaceutics and pharmacokinetics, the best results are obtained by using chromatographic techniques applied to chiral columns.[13,87]

Tandem mass spectrometry: There are several advantages to triple quadrupole mass spectrometry compared to previous analytical methods: [14]

- A high degree of specificity
- High sensitivity, ranging from pg•mL−1 to ng•mL−1
- It takes about 4–6 minutes to run

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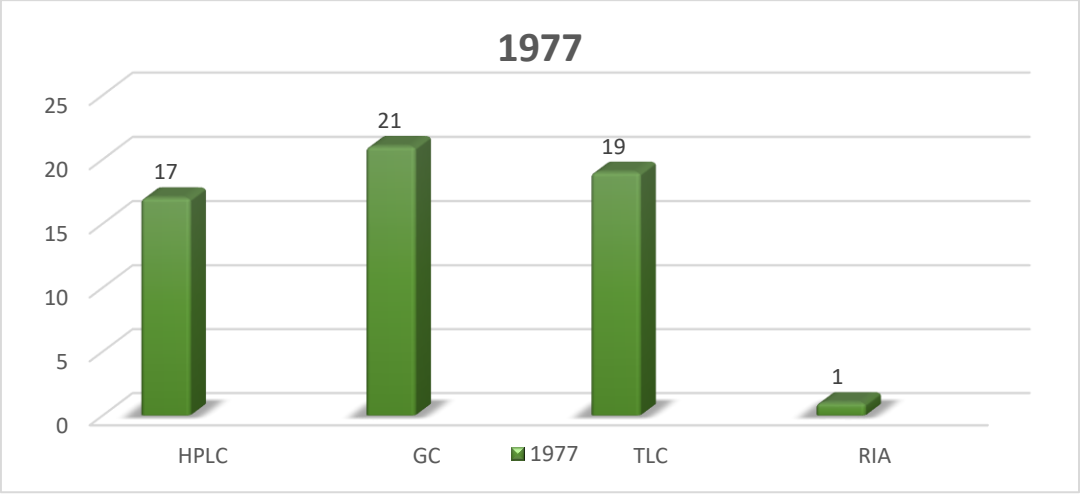
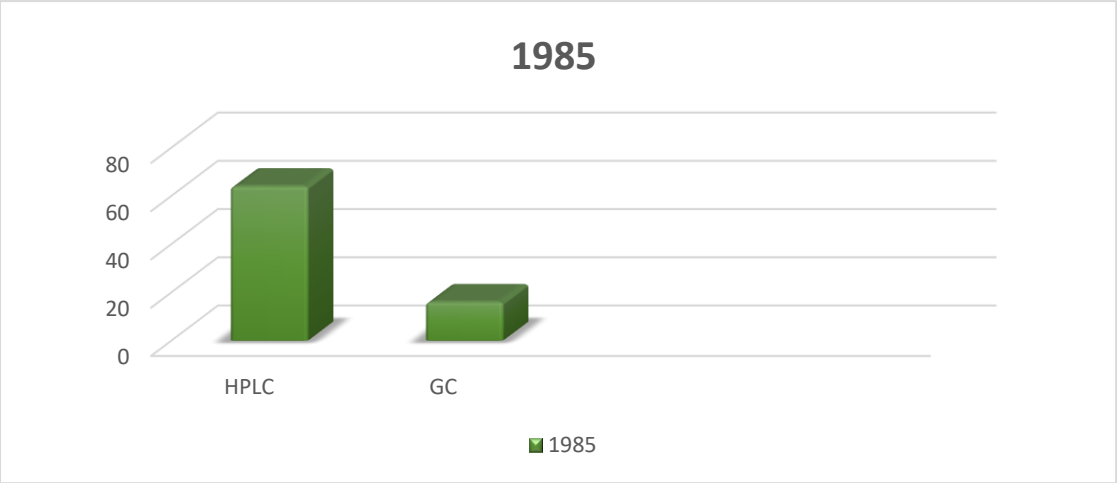
As a detector for liquid chromatography (LC-MS-MS), triple quadrupoles are most useful. A variety of drugs have been bioassayed in biological fluids using this technique, including drugs with high distribution volumes that require high sensitivity to estimate plasma concentrations of pg•mL−1. The mass spectrometry technique can also be used to detect gas chromatographic eluate, although it is less commonly used for this purpose.[15] The following tables show the comparison of conditions used to perform a bioassay using a combination of chromatographic techniques (HPLC and GC), a tandem mass spectrometry technique, and the 4hydroxy metabolite of nimesulide in human plasma and isosorbide-5-mononitrate. Tandem mass spectrometry has several advantages, namely the run time is shorter by 3 to 5 times, the LLOQ sensitivity is 200 to 300 times better than traditional mass spectrometry, and the period required to perform the bioassay 1000 analyses was 3 to 4 times shorter than traditional mass spectrometry [16] Based on the characteristics outlined above, it has become one of the most widely used methods in the fields of pharmacokinetics, bioavailability, and bioequivalence [17]





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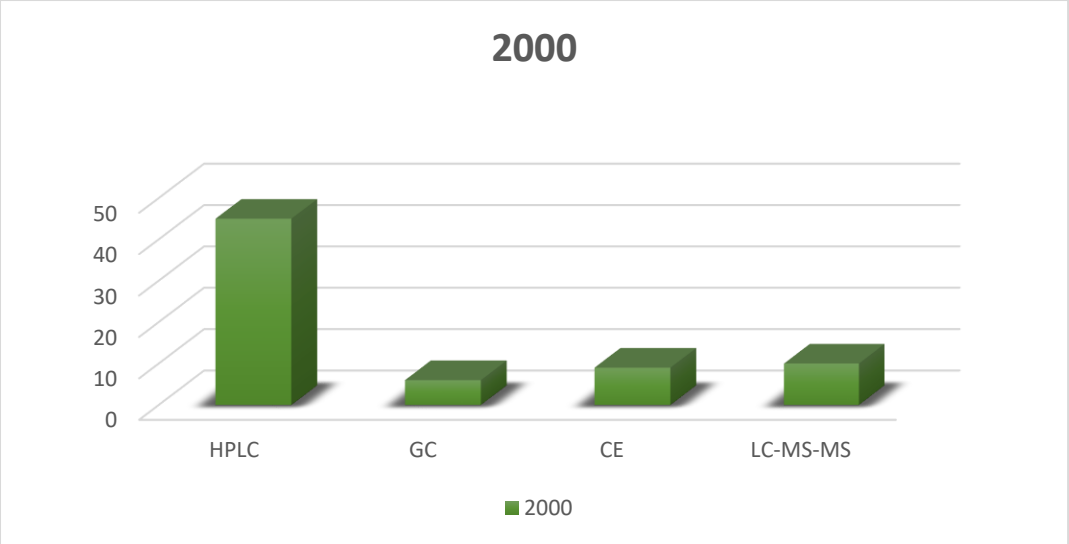

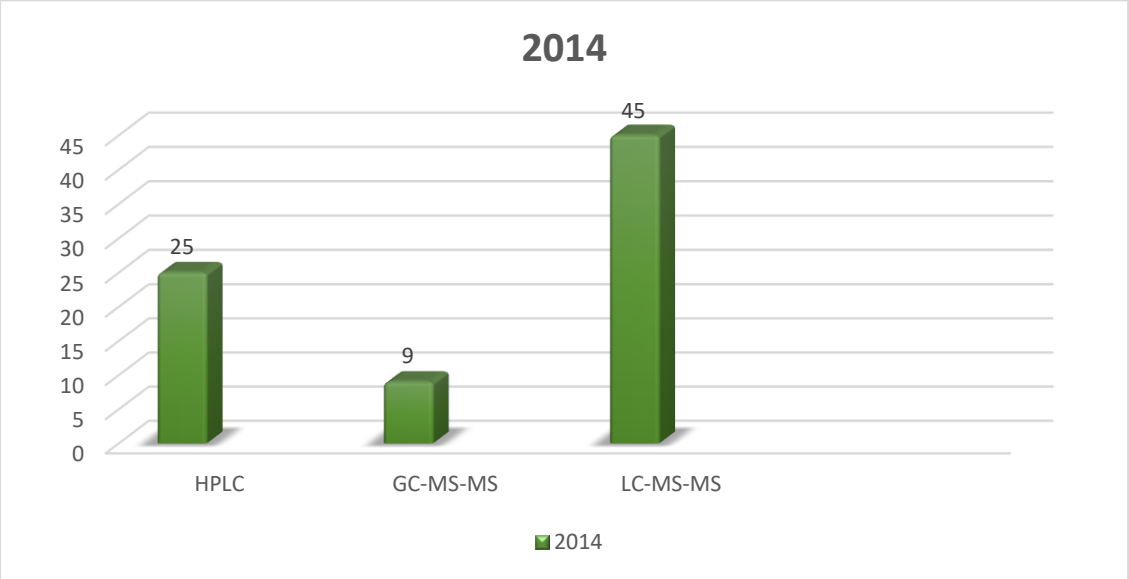

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This figure illustrates how analytical techniques for bioassays of drugs in biological fluids have developed since 1977

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1 **Bioequivalence and bioavailability guidelines for the EU:** In 1991, a guideline was published by the European Union on BAs and BEs. An updated version of the BA and BE guideline was released ten years later in 2010.[18] It should be noted that the last guideline, which was followed by the current guideline, covered only immediate release formulations. A draft guideline on modified release formulations was developed in 1996 and updated in 2013. As you can see from the above guidelines, a lot of emphasis was put on the most common problems that occur in BA and mainly in BE trials.[19]

2 **Guidelines focused on problems**

- **Titre difference between test and reference*:*** When conducting bioequivalence trials, the difference between reference and test titers cannot exceed 5%. Cmax and AUC will need to be checked using dose-normalized parameters if the difference between BEs is greater.[20] A dose normalization like the one above was not allowed in previous EMA guidelines. Pharmaceutics can accept drugs in a titer range greater than 95.0–105.0% when this possibility is available.[21]

- **Study carryover from pre-dose to post-dose:** When volunteers enter the crossover trial, their drug concentration can be measurable above the LLOQ. There is a possibility of statistically processing the volunteer if this value is at least 5% of Cmax. It is mandatory to exclude a given volunteer from statistical analysis if the baseline level is greater than 5% of Cmax. According to the guidelines of the US Food and Drug Administration, this statement is accurate.[22]

- **90 percent confidence interval widened:** Cmax In the first EU guideline, it was allowed to expand by 90% the stated 0.80 – 1.25 confidence intervals (CIs) for assessing BE for Cmax, which was originally allowed to be expanded from the 0.80 – 1.25 interval but without specifying a maximum measurement [23] It was determined from publications that the expanded limit would be between 0.70 and 1.43. As per the EU guideline edited in 2001, when there is high variability of Cmax, it is possible to enlarge the 90% CI of the Cmax by 0.75 to 1.33. By contrast, the last EU guideline31 allowed enlargement of the 90% confidence interval only if the intrasubject coefficient of variation (CV) is greater than 30%, a replicate design is used, and only for oral formulations that are also immediate-release formulations as shown in the table below.

  To support the statistical interpretation, the FDA guidelines on bioequivalence prohibit enlarging the 90% CI of the Cmax beyond 0.80 to 1.25. To be considered bioequivalent, Cmax must fall within the gap of 0.90–1.11 for narrow therapeutic index drugs (NTIDs) with a Cmax that is particularly important to safety, efficacy, and level monitoring [24].

  When Cmax is important to safety, efficacy, or drug level monitoring (NTIDs), drugs with narrow therapeutic indices Cmax should produce an acceptance interval of 0.90–1.11.

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Table 3: For different CV percent values, the 90% CI of Cmax has been enlarged.

Percentage of CVs within subjects	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

- **Parent drug or active metabolite(s):** A recent EMA guideline clarifies that for parent drugs and active metabolites, the bioassay and pharmacokinetic assessment should be carried out only on their parent drugs. As a result, it can be difficult to follow the plasma concentration of the parent drug in some cases, since it disappears quite rapidly, whereas in the case of the active metabolite, its plasma concentration is evidently shaped with a well evident shape. In the following table, you will find a list of a number of parent drugs that are largely bio transformed into active metabolites Betahistine is an example of a non-active metabolite that requires bioequivalence assessment, even if it is not active.[25-26]

- **Waivers for in vivo bioequivalence:** There are some specific cases in which the guidelines for the EU and the US allow the waiver of the in vivo bioequivalence test to be performed. Water-based solutions can usually be approved based on a BE trial of just one dose, usually the most effective, but sometimes the least effective. The highest dose can sometimes be approved, sometimes the least effective. Intravenous (IV) injectable solutions are not covered by the BE. During the past few years, there has been an increase in the number of drugs that fall into group 1 or group 3 of the BCS. Table 6 shows this. Class 1 and 3 drugs are eligible for a BE exemption, being classified in the BCS as 1 and 3. The number of drugs classified in the two classes that have been discussed above has led to a reduction in the need for BE trials. At the requested pH ranges, several studies have been conducted in vitro to determine the solubility of the polymers and their rate of dissolution.[27-29]

  Currently, guidelines fail to address the following problems:Multiple peak phenomenon. It is possible for drug plasma concentrations to peak twice or more after absorption, depending on the drug. When drugs enter the enterohepatic circulation, there is a two-peak phenomenon. Two to twelve hours after administration, the first peak occurs, and the second peak occurs between six and twelve hours later. Mycophenolic acid, ursodeoxycholic acid, piroxicam, glibenclamide, and its parent drug, glibenclamide, show this effect.[30-33]

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Active metabolite	Parent drug	AUC ratio between metabolites and parent drugs
Enalaprilat	Enalapril	2.5[75]
Zofenopril at	Zofenopril	6.7[75]
Hydroxyflutamide	Flutamide	6076
Hydroxypurinol	Allopurinol	4477
Acid metabolite of terfenadine	Terfenadine	⩾100[78]
Monohydroxycarbamazepine	Oxcarbazepine	⩾100[79]
Mycophenolic acid	Mycophenolatemofetil	⩾2552,80
Desmethylclozapine N-oxideclozapine and other metabolites	Clozapine	~70% metabolized[81]

As a result, other cases of sudden and rapid peaks occur, such as when the second peak or a series of peaks appears within 3 to 4 hours after the dosing, which are more complex and less predictable. In these cases, one of the most memorable incidents would be what happened to a person who took diclofenac. Various authors have identified a tetrahydro-diclofenac as the result of diclofenac's hydration during the process of absorption through the gut, where it is metabolised into less soluble tetrahydrodiclofenac. As soon as diclofenac was incorporated into a formulation that was able to be absorbed very quickly, it was found that only one peak of the relevant entity, namely the first peak, would appear.[34]There are some situations in which Marzo and Reiner recommend that bioequivalence be assessed only on the value of the 90% CI of AUC, while Cmax should be evaluated based on whether the individual value for the given dose lies within the expected range of the previous literature, so as to exclude any need for a reassessment or a change in the dose of the drug. According to EMA, this suggestion was not taken into consideration, neither in the guidelines nor in the questions answered during the meeting [35-37].

- **Concerns about bioequivalence from an ethical perspective*:*** In the course of a bioequivalence trial, some drugs may cause safety problems when administered to healthy volunteers as part of the trial. Considering the inability to administer cytostatic agents to healthy volunteers, it is important to also consider the following cases as a result of the inability to administer cytostatic agents to healthy volunteers.[38] There are other drug interactions as well, such as cyclosporine, which may affect renal clearance and flutamide, which may make a man's gynecomastia worse over a period of about 30 days. It has been found that both women and men who take morphine have a number of adverse effects.[39-41] In order for warfarin to reach its steady state, it takes a long period of treatment and clozapine has significant adverse effects on men when it comes to achieving it In

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the above cases, as well as in many similar situations, it is important to carefully consider and avoid the administration of the aforementioned drugs, and, of course, in any similar circumstances in the future, especially in clinical trials involving repeated dose regimens to reach a steady state in healthy volunteers.[42]The operating guidelines require assessing the BE of drugs with a long half-life that are administered in long-acting oral formulations, transdermal delivery systems, or immediate release formulations.[43]

- **Baseline substances and their endogenous counterparts:** Endogenous substances are typically exposed to very complex problems when it comes to their PKs, BAs, and BEs, which are not properly addressed by the EU operating guidelines. This substance presents the following challenges when managing trials:

  - Certain hormonal levels, such as melatonin, cortisol, and female sexual hormones, fluctuate around an average level and have certain rhythms. [44-46]
  - In the above scenario, the European Medicines Agency (EMA) seeks to subtract baseline, which can be particularly challenging when dealing with endogenous rhythm;
  - The body has a variety of homeostatic equilibrium mechanisms that work to maintain an equilibrium so that excessively high or low concentrations of endogenous substances in the blood do not occur;[47]
  - Dilution of the systemic circulation by a relatively low amount that enters the blood and thereby diluted by a larger amount that contains the requisite information;
  - With endogenous substances, there is a multicomponent reversible metabolism
  - In the body, bones, fats, and red blood cells are examples of specific body stores;[48]
  - As a result of the renal threshold, several substances, such as L-carnitine, most ions, and a small number of amino acids, can be maintained at constant concentrations in the blood system.[49]

Consequently, the absorption of exogenous substances administered endogenously doesn't produce a well-defined plasma concentration shape because of these homeostatic mechanisms. The problem in these cases is that the regulatory request to subtract baseline can lead to increased variability even after a peak shape is obtained, as can be seen from the simulation which is reported below as an example, even after the peak shape is obtained. [50-54]

AUC (total value 100 $ng \cdot ml^{-1} \cdot h \pm 20$ CV = 20 % measured)
AUC baseline 80 $ng \cdot ml^{-1} \cdot h \pm 8$ CV = 10 %
AUC net value (total –20 $ng \cdot ml^{-1} \cdot h \pm$ 20 CV = 100 % baseline) 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
$= 392 \cdot CV^2$

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$= 392 \cdot 1^2$
$= 392 \cdot 1$
$= 392$ subjects

Scheme 1: Pool size evaluation of an endogenous substance without and after baseline subtraction

In simulations, when 100,000 IU (2.50 mg) of cholecalciferol were administered orally daily for 7 days, the peak of the drug was reached after 7 days, and the baseline was restored after 100 days. As a result of the above considerations, it appears that in most cases the baseline subtraction should be avoided. [55] The cumulative urinary excretion of drugs such as L-carnitine, most ions, and some amino acids can provide a better estimate of bioavailability than the profile of plasma concentrations when assessing the bioavailability of these drugs. A more complex solution is required when endogenous substances are biotransformed.[56] In some cases, there is a possibility of avoiding the subtraction by administering high doses of the drug, resulting in the presence of a clearly identifiable peak shape. Other times, if the exogenous administration was repeated over a relatively long period of time, it may be sufficient to consider the baseline as a product of the exogenous administration during the repeated regimen.[57-59] Testing against a reference population in a phase III trial is another option for the Test Vs. Reference comparison. Potassium, levothyroxine, and omega 3 derivatives are a few of the specific cases monitored by regulatory authorities particularly closely.[60] In the case of potassium, the US Food and Drug Administration (FDA) guidelines suggest that bioequivalence be assessed on the basis of urinary excretion of this ion, and the plasma level of this ion should not be considered to be an indication of its bioavailability as it is not indicative of it.[62] Specifically, a guideline edited by the US food and drug administration recommends that when treating patients with levothyroxine, the dosage should be high enough to produce a relevant peak shape, without subtracting the baseline dose. Eicosatetraenoic acid and docosahexaenoic acid are omega 3 derivatives contained in the soft gelatin capsule, which is an exemption because it contains only these two drugs without any excipients, according to the EU EMA Questions and Answers document.[63]

- **Methods for pre-analytical preparation of chromatographic bioassay samples*:*** A LC-MS-MS and GC experiment requires extractions of liquids and solids and internal standards. Deuterated analytes are usually the best ISs for LC-MS-MS, since they are chemically identical to the true analytes, but can be detected by a multiquadrupole instrument.[64-66] Among the relevant aspects that need to be considered by the analyst are the following:

  - Distribution of analytes in red cells in plasma. The procedure should be focused on the analyte when it has an asymmetric distribution, e.g. bioassaying the whole blood for the analyte;
  - Both parent drug and metabolite must be tested if reversible metabolism is occurring; alternatively, one compound must be chemically transformed into two or more other compounds;[67]
  - A planned time, the shorter the better, must be set for centrifuging tubes containing blood in the presence of the parent drug's hydrolytic metabolism,

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followed by freezing in ice-dry and storage in the freezer. One example is acetylsalicylic acid; [68-69]

- As soon as the analytical procedure has been validated, the anticlothing agent should be selected and maintained throughout the next stage of bioassays of the samples [70].

IV. GUIDE TO VALIDATION OF ANALYTICAL RESULTS

In 1992, Shah et al. published the first guidelines for managing and validating bioanalytical methods. After a decade, the same authors edited their previous recommendations.[70] The US FDA34 and the EU developed specific guidelines for analytical validation.[71-74] The above guidelines concentrated on directions for describing detailed pre-study validation procedures in a Validation Report and for documenting study specific validation in an Analytical Report. Question and Answers also discuss incurred sample reanalysis. [75]

V. ADJUNCTIVE REMARKS

The following are some additional things that should be taken into consideration while organising pharmacokinetic research for regulatory authorities. This literature review reveals that many waivers do not allow the use of medications that have relatively short therapeutic windows. However, because there is no clear meaning of the term, putting it into practise might be challenging. Because repeated dosage designs might potentially cause tolerability problems and are often required by operating requirements, it is best to steer clear of them whenever it is possible to do so. We review the ethical considerations that are highlighted by such studies, and we do it with the help of numerous healthy volunteers. The regulations concerning bioequivalence make no mention of this subject at all. [76-77] In applications that do not require bioavailability but only require local therapy for local action, pharmacokinetic (PK) investigations in healthy volunteers should be used rather than phase III clinical trials. According to what I've observed, the coefficients of variance in some test-reference comparisons are so high as to render any bioequivalence assessment worthless. This is the conclusion I've drawn from the data. The relatively large CV made it difficult to calibrate the appropriate dose of test and reference. The challenge was caused by the fact that. When we assessed the local and systemic effects of these topical treatments in healthy volunteers over the course of a full treatment cycle (test vs. reference), we discovered that they were well tolerated. This was the conclusion we reached when we compared the test group to the reference group. Before this, for a period of three years, the AIFA in Italy used this framework for the purpose of evaluating BE. Even if a Phase III clinical study is not always required to determine the bioequivalence of a topical medication, the EU might take into consideration employing this method in some circumstances. [78]

VI. DISCUSSION

New drug applications require extensive screening of thousands of compounds, which is a part of modern drug discovery research (NDA). When dividing molecules into the four categories of the Amidon BCS, scientists must use PKs from the very beginning of the screening process. [79] For the past two decades, there has been no applied research

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into novel uses for patented pharmaceuticals because no new chemical entities have been developed. Drugs like nifedipine, with a half-life of around two hours, can be given just once a day thanks to extended-release oral forms. Mesalamine and other slow-acting formulations need the ileo-cecal valve to open before they can begin to work. [80] Ability to circumvent the first-pass effect, which, in the case of drugs like nitroglycerin, metabolises a sizable fraction of the substance before it reaches the body (and hence reduces its therapeutic efficacy). In addition to clonidine and female sex hormones, TDS also creates nicotine formulations. [81-82] To prolong the drug's effects for up to a month, some doctors recommend injecting a depot form of the medication into the muscle, subcutaneous tissue, or fat. Further, novel fixed-dose combinations and dispersible formulations are required. The aforementioned applications were usually derived from BE data analysis in accordance with ANDA procedures, which exempted a drug from targeted population clinical trials on the basis of the already established data concerning the drug's activity and safety, compared with a reference formulation in test form. [83] Recent years Analytical Quality by Design (AQbD) through Design of experiment (DoE) is an emerging approach relied on systematic, holistic, robust and homogenous product development. [84, 85] The QbD based software approach is also quite benefits for faster optimization, analysis of various samples, indent quantification, error-free revalidation, saving of reagents and resources [86]. This holistic approach is broadly used current scenario for analytical, bioanalytical, biotechnological and forensic applications, as well as to achieve regulatory flexibility. [86,87] The principle of QbD to analytical and bio-analysis can be implemented for biological sample analysis in chromatographic techniques like HPLC, UFLC, and modern sophisticated hyphenated techniques Like LC-MS, LC-MS-MS etc. [85-88] However, the LC-MS-MS methodology, is the most applicable analytical method, & is focus of application. It is fundamental to a primary approach to pharmacokinetics. Both the Food and Drug Administration (FDA) of the United States and the European Medicines Agency (EMA) standardized and regulated these differences and waived some *in-vivo* bioequivalence standards when *in-vitro* data on solubility, dissolution, and disaggregation were available for most cases. One of the most pressing unanswered challenges is how to derive net plasma concentrations by subtracting the effects of exogenous drugs from the homeostatically controlled amounts used as a reference. It might be challenging to interpret recorded amounts when exogenous chemicals are subtracted from baseline concentrations. [89]

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