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Review

A systematic and critical review on bioanalytical method validation using the example of simultaneous quantitation of antidiabetic agents in blood

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ABSTRACT

A systematic and critical review was conducted on bioanalytical methods validated to quantify combinations of antidiabetic agents in human blood. The aim of this article was to verify how the validation process of bioanalytical methods is performed and the quality of the published records. The validation assays were evaluated according to international guidelines. The main problems in the validation process are pointed out and discussed to help researchers to choose methods that are truly reliable and can be successfully applied for their intended use. The combination of oral antidiabetic agents was chosen as these are some of the most studied drugs and several methods are present in the literature. Moreover, this article may be applied to the validation process of all bioanalytical

1. Introduction

The success of a therapy depends on the mechanism of action of the drug, patient adherence, and appropriate levels of the drug in the bloodstream. The latter two factors can be evaluated through bioanalytical methods, which are employed to determine drug and/or metabolite concentrations in biological matrices. These analyses play a significant role in pharmacokinetic, pharmacodynamic, therapeutic drug monitoring (TDM), bioavailability, bioequivalence, and toxicology studies [1–3].

In order to ensure that a method is suitable for its purpose, it must be experimentally validated. Specific guidelines for bioanalytical method validation have been elaborated to advise analysts on this process, such as Food and Drug Administration (US FDA) [3], European Medicines Agency (EMA) [4] and *Agência Nacional de Vigilância Sanitária* (ANVISA) [5] guidelines. In the case of TDM, there are some specific guidelines, such as the American Association for Clinical Chemistry TDM generic assay validation guidance and FDA guidance for some specific TDM assays, which mainly target immunoassay tests. Despite these guidelines, several records present problems during validation, which may affect the results and, consequently, the intended application. In this article, the authors conducted a systematic review on validated bioanalytical methods to quantify drugs in human blood. The combination of antidiabetic agents was chosen, since type 2 diabetes is one of the most important health problems in the world and many bioanalytical methods are available in the literature [6].

To date, no systematic review has been performed to collect information on the various validated methods to quantify antidiabetic agents in human blood. In the present study, we systematically selected records on this subject and verified whether they were properly validated, pointing out and discussing the main problems in the validation process.

2. Methods

2.1. Systematic literature search

A systematic review of the literature involving validated methods for quantitation of oral antidiabetic agent combinations in human blood was conducted. Systematic reviews guarantee that all publications on a particular subject will be included in the review. No restrictions regarding the analytical technique or publication date were imposed. For the search strategy, the descriptors were employed: “oral antidiabetic*” and generic and brand name of the drugs acarbose, acetohexamide, alogliptin, buformin, canagliflozin, carbutamide, chlorpropamide, dapagliflozin, empagliflozin, glibenclamide, glibornuride, gliclazide, glimepiride, glipizide, gliquidone, glisoxepide, glyburide, glycopyramide, linagliptin, metformin, miglitol, nateglinide, pioglitazone, repaglinide, rosiglitazone, saxagliptin, septagliptin, sitagliptin, teneligliptin, tolazamide, tolbutamide, vildagliptin, and voglibose; “valida-

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tion", "quantitation", "therapeutic monitoring", "plasma", "blood", "serum", "human plasma", "human blood", "human serum" and the name of each analytical technique, combined with the Boolean operators "AND" and "OR". The search was conducted in the Medline, Web of Science and Scopus databases and was completed in October 2015. In addition, reference lists of the records were searched manually to retrieve any further articles. Records in non-Roman characters were excluded from the systematic review.

2.2. Study selection

Initially, two reviewers independently selected studies based on their title and abstract (screening), with disagreement being adjudicated by a third reviewer. Articles that appeared to be potentially relevant were fully analyzed by the same reviewers.

2.3. Data extraction and analysis

The collected data included all validation data (selectivity, linearity, precision, accuracy, limit of detection, limit of quantitation, matrix effect, carryover, stability, robustness, recovery, system suitability and the guideline followed). The validation parameters were discussed and evaluated according to the most complete guidelines for bioanalytical method validation currently available: Guidance for Industry – Bioanalytical Method Validation by US FDA [3], the guideline on bioanalytical method validation by EMA [4] and Brazilian RDC 27 from May 17, 2012 – Minimum requirements for validation of bioanalytical methods by ANVISA [5].

3. Results and discussion

A total of 2962 records were identified initially. After screening, 2872 references were excluded, and after full-text analysis, 56 records were removed. Therefore, 34 articles [7–40] were included and no further articles were identified through the manual search. A flow chart of the selection process is presented in Fig. 1.

The main reasons for excluding articles were that validation was performed in solution [41–52] and method development without validation [53–72]. Validation in solution with subsequent extrapolation to biological samples is not encouraged due to the matrix effect, a factor that should be considered when developing a bioanalytical method [3–5] and that will be further discussed in this review. This miscomprehension about method extrapolation was probably because some researchers followed the guideline from the International Conference on Harmonisation (ICH) [73], which covers only analytical methods, or because the authors did not follow any guideline at all. Furthermore, some methods had been developed but not validated. We observed two reasons for the absence of validation data in the articles: the study was performed before the guidelines had been published [53–66]; and the study focus was on method development and validation was not addressed [67–72]. Other reasons for excluding articles were: non-simultaneous method [74–77], inappropriate sample preparation [78,79], literature review [80], animal plasma sample [81], language in non-Roman characters [82–84], validation of only one analyte [85,86], and pharmacokinetic studies (PK) that used a previously developed and validated method [87–97].

3.1. Oral antidiabetic agent combinations

The methods included in this review covered a total of 24 antidiabetic agents from six classes: buformin, carbutamide, chlorpropamide, glibenclamide, glibornuride, gliclazide, glimepiride, glipizide, gliquidone, glisoxepide, linagliptin, metformin, miglitol, nateglinide, phenformin, pioglitazone, repaglinide, rosiglitazone, saxagliptin, sitagliptin, teneligliptin, tolazamide, tolbutamide, and vildagliptin.

The association analyzed in each method can be found in Table 1, along with other general characteristics of the validation of methods.

3.2. Bioanalytical method validation

The methods included in this systematic review were validated according to the criteria established by the "Guidance for Industry –

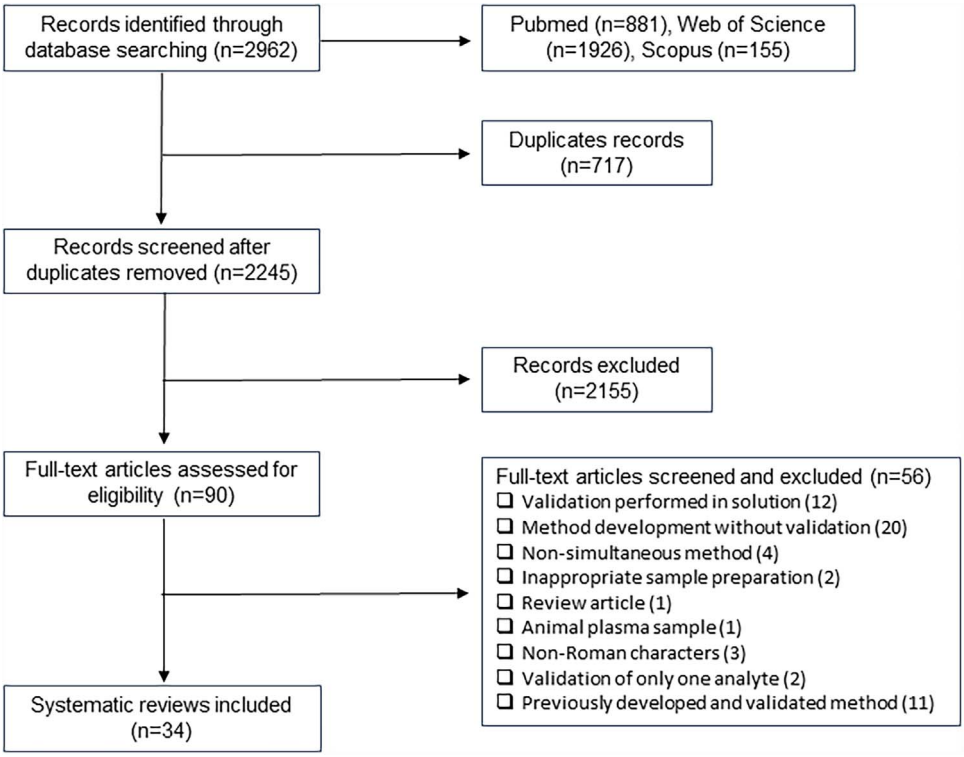

Fig. 1. Flow chart of the systematic review.

Table 1
General characteristics of the included records.

Record	Analytes	Analytical technique	Limit of detection/quantification (ng mL^{-1})	Linearity	Method of recovery evaluation	Recovery (%)[a]
AbuRuz et al. [7]	GBC, GCZ, GMP, GPZ and MET	HPLC (UV)	LOD/LLOQ: GBC = 4/7, GCZ = 13.5/22.5/ GMP = 4/7.5/GPZ = 4.5/7.5 and MET = 3/5	–	Not reported	GBC: 81.8–87.3; GMP: 94.7–101.9; MET: 76.3–89.7; GCZ: 87.6– 97.7; GPZ: 79.7– 90.4; MET: 87.8– 97.1
Agrawal et al. [8]	GCZ and MET	SFC–MS/MS	LLOQ: GCZ = 7.5 and MET = 7.5	GCZ = 7.5–7000 and MET = 6–3,550 ng mL^{-1}	Not reported	GCZ: 93–96; MET: 95–102
Attimarad et al. [9]	MET and MIG	HPLC–MS	LOD/LLOQ: MET = 3.83/10.82 and MIG = 7.08/22.91	MET = 20–2,000 and MIG = 25–4,000 ng mL^{-1}	Spiked matrix before SP × spiked matrix after SP	MET: 95.4; MIG: 94.9
Ben-Harder et al. [10]	MET and ROS	HPLC (UV)	LOD/LLOQ: MET = 0.12/1 and ROS = 0.36/3	MET = 5–2.500 and ROS = 1–500 ng mL^{-1}	Not performed	Not performed
Binz et al. [11]	GBC, GCZ, GPZ, GQD and MET	HPLC–MS/ MS	LLOQ: GBC and GPZ = 5, GCZ, GQD and MET = 50	GBC and GPZ = 0–100, GCZ, GQD and MET = 0–1,000 ng mL^{-1}	Spiked matrix before SP × spiked matrix after SP	GBC: 61.6; GCZ: 56.4; GPZ: 67.6; GQD: 61.0; MET: 61.7
Chen et al. [12]	MET and ROS	HPLC–MS/ MS	LLOQ: MET = 4.040 and ROS = 1.054	MET = 4.040–5,050 and ROS = 1.054–263.5 ng mL^{-1}	Spiked matrix before SP × spiked matrix after SP	MET: 87.0–93.6; ROS: 80.5–92.0
Di Rago et al. [13]	GBC, GCZ, GMP, GPZ, MET, PIO, ROS and SIT	HPLC–MS/ MS	LLOQ: GBC = 50, GCZ and MET = 500	GBC = 50–2,000, GCZ = 500–1,000, GMP = 50–400, GPZ = 500–1,000, ROS = 500–1,000[1] and SIT = 50–400 ng mL^{-1}	Spiked matrix before SP × spiked matrix after SP	GBC: 91–105; GCZ: 80–93; GMP: 102–106; GPZ: 95–105; MET: 74–94; ROS: 98–123; SIT: 95–116
Ding et al. [14]	GPZ and MET	HPLC–MS/ MS	LLOQ: GPZ = 4 and MET = 20	GPZ = 4–800 and MET = 20–4,000 μg mL^{-1}	Not performed	Not performed
Georgita et al. [15]	GBC and MET	HPLC–MS/ MS	LLOQ: GBC = 4 and MET = 40	GBC = 5–400 and MET = 50–2,000 μg mL^{-1}	Spiked matrix before SP × solution	GBC: 73.4; MET: 56.0
Gonzalez et al. [16]	CAR, GBC, GBN, GCZ, GMP, GPZ, GQD, MET, REP, ROS, TZD and TBT	HPLC–MS/ MS	LOD/LLOQ: CAR = 0.32/1.13, GBC = 0.18/0.62, GBN = 0.25/0.48, GCZ = 0.61/1.46, GMP = 0.20/0.6, GPZ = 0.24/0.69, GQD = 0.39/0.61, MET = 0.76/0.84, REP = 0.20/0.73, ROS = 0.20/0.77, TZD = 0.47/1.20 and TBT = 0.57/2.52	GBC, GBN, GCZ, GMP, GPZ, GQD, MET, REP, ROS and TBT = 120–30,000 CAR and TZD = 25–1,880 000 μg mL^{-1}	Spiked matrix before SP × spiked matrix after SP	CAR: 66.6; GBC: 62.0; GBN: 63.6; GCZ: 39.0; GPZ: 74.5; GQD: 48.2; MET: 65.1; REP: 58.7; ROS: 57.5; TZD: 47.3; TBT: 65.8
Hefnawy et al. [17]	GMP and ROS	CE (DAD)	LOD/LLOQ: GMP and ROS = 20/50	GMP and ROS = 0.05–16 μg mL^{-1}	Spiked matrix before SP × solution	GMP: 97.2–100.6; ROS: 98.7–101.9
Hess et al. [18]	GBC, GBN, GCZ, GMP, GPZ, GQD, GSX, NAT, PIO, REP and ROS	HPLC–MS/ MS	LOD/LLOQ: GBC, NAT, PIO,REP and ROS = 1/1 GBN,GCZ, GMP, GPZ, GQD, GSX,= 1/5	GBC = 1–200, GBN, GQD, GXS = 10–1,000, GCZ = 10–5,000, GMP = 10–3,000, GPZ = 5–1,000, NAT, PIO, REP and ROS = 1–500 ng mL^{-1}	Spiked matrix before SP × spiked matrix after SP	GBC: 84.02–101.4; GBN: 95.60–98.58; GCZ: 79.68–88.44; GMP: 85.05–99.57; GPZ: 79.32–102.4; GQD: 89.63–90.59; GSX: 90.10–101.4; NAT: 89.89–97.92; PIO: 90.55–103.8; REP: 99.98–102.8; ROS: 91.26–105.7; SAX: 37.20–39.20; SIT: 79.90–85.25; VDP: 40.62–41.87
Hoizey et al. [19]	CAR, CHL, GBC, GBN, GCZ, GMP, GPZ and TBT	HPLC–MS/ MS	LOD/LLOQ: CAR and CHL = 1.98/31.25, GBC = 0.24/3.9, GBN and GPZ = 1.95/ 7.81, GCZ = 0.49/7.81, GMP = 0.98/15.6 and TBT = 4.9/78.1	CAR and CHL = 0.03125–2/GBC = 0.00391–0.25/ GBN, GCZ and GPZ = 0.00781–0.5/GMP = 0.0156–1/ TBT = 0.0781–5 μg mL^{-1}	Spiked matrix before SP × solution	CAR: 81.5–107.2; CHL: 97.0–118.2; GBC: 95.1–101.9; GBN: 83.0–98.4; GCZ: 102.2–110.9; GMP: 92.9–109.9. GPZ: 94.3–110.4; TBT: 92.9–98.7
Jagadeesh et al. [20]	MET and PIO	HPLC–MS/ MS	LLOQ: MET = 25.01 and PIO = 15.04	MET = 25–3000 and PIO = 15–2500 ng/mL^{-1}	Spiked matrix before SP × solution	Hydroxypioglitazone: 71.35–74.76; MET: 84.22–85.97; PIO: 74.05–81.10
Jingar et al. [21]	GMP and ROS	HPLC (UV)	LOD/LLOQ: GMP = 20/41.146 and ROS = 20/41.821	GMP = 41.066–2094.94 and ROS = 40.994–2007.556 ng mL^{-1}	Spiked matrix before SP × solution	GMP: 65.7–74.3; ROS: 65.2–70.5
Li et al. [22]	GPZ and MET	HPLC–MS/ MS	LLOQ: GPZ = 2.55 and MET = 4.10	GPZ = 2.55–408 and MET = 4.10–656 ng mL^{-1}	Spiked matrix before SP × spiked matrix after SP	GPZ: 83.5–94.9; MET: 78.9–85.3
Lin et al. [23]	GPZ and ROS	HPLC–MS/ MS	LLOQ: GPZ and ROS = 1	GPZ and ROS = 1–2000 ng mL^{-1}	Spiked matrix before SP × solution	GPZ: 85.2–86.7; ROS: 84.3–93.5
Magni et al. [24]	CHL, GBC, GPZ and TBT	HPLC–MS	LOD/LLOQ: CHL, GBC, GPZ and TBT = 2/ 10	CHL, GBC, GPZ and TBT = 2–100 ng mL^{-1}	Not reported	CHL: 94.8; GBC: 88.6; GPZ: 90.0

(continued on next page)

Table 1 *(continued)*

Record	Analytes	Analytical technique	Limit of detection/quantification (ng mL^{-1})	Linearity	Method of recovery evaluation	Recovery (%)[a]
Maier et al. [25]	GBC, GCZ, GMP, GPZ, REP and TBT	CE (DAD)	LOD/LLOQ: GBC = 8.9/28.8, GCZ = 4.6/15.4, GMP = 3.1/10.5,GPZ = 6.9/15.4, REP = 2.5/8.4 and TBT = 2.3/7.8	GBC, GCZ, GMP,GPZ, REP and TBT = 5–200 nmol.L^{-1}	Not performed	Not performed
Maurer et al. [26]	GBC, GBN, GCZ, GMP, GPZ, GQD, GSX, TZD and TBT	HPLC–MS	LOD/LLOQ: GBC = 2/10, GBN and GQD = 30/300, GCZ = 50/500, GMP = 10/100, GPZ and GSX = 5/50, GQD = 30/300 TZD and TBT = 3000/30000	GBC = 0.01–0.2, GBN = 0.3–1.1, GCZ = 0.5–6, GMP = 0.1–3, GPZ = 0.05–2, GQD = 0.3–1.1, GSX = 0.05–1, TZD and TBT = 30–120 μg mL^{-1}	Spiked matrix before SP × solution	GBC: 79.0–100.1; GBN: 68.0–82.4; GCZ: 67.8–69.2; GMP: 76.2–76.4; GPZ: 72.3–74.1; GQD: 82.6–96.4; GSX: 65.9–71.4; TZD: 76.6–79.1; TBT: 72.0–83.4
Mistri et al. [27]	GBC and MET	HPLC–MS/MS	LLOQ: GBC = 5 and MET = 20	GBC = 5–500 and MET = 20–2,500 ng mL^{-1}	Spiked matrix before SP × spiked matrix after SP	GBC: 71.2; MET: 69.3
Ni et al. [28]	GMP and PIO	HPLC–MS/MS	LLOQ: GMP and PIO = 0.2	GMP = 0.2–250 and PIO = 0.2–1250 ng mL^{-1}	Spiked matrix before SP × solution	GMP: 77.11–78.53; PIO: 83.41–88.51
Pontarolo et al. [29]	MET and VDP	HPLC–MS/MS	LOD/LLOQ: MET = 1.5/5 and VDP = 0.75/5	MET and VDP = 5–500 ng mL^{-1}	Spiked matrix before SP × spiked matrix after SP	MET: 89.2–93.0; VDP: 103.2–107.3
Ranetti et al. [30]	GCZ and MET	HPLC (UV)	LLOQ: GCZ = 49/MET = 50	GCZ = 49–4,875 and MET = 50–5,000 ng mL^{-1}	Not performed	Not performed
Rashid et al. [31]	GBC and MET	HPLC (UV)	LOD/LLOQ: GBC = 2/4 and MET = 28/30	GBC = 5–625 and MET = 30–625 ng mL^{-1}	Spiked matrix before SP × solution	GBC: 98.19–98.56; MET: 98.51–99.26
Reddy et al. [32]	MET and SIT	HPLC–MS/MS	LOD/LLOQ: MET = 2.5/10 and SIT = 0.75/3	MET = 10–2,206 and SIT = 3–800.5 ng mL^{-1}	Spiked matrix before SP × solution	MET: 89–94; SIT: 100–107
Sengupta et al. [33]	GMP, MET and PIO	HPLC–MS/MS	LLOQ: GMP and PIO = 2.5 and MET = 10	GMP = 2.5–500, MET = 10–1500 and PIO = 2.5–1,000 ng mL^{-1}	Spiked matrix before SP × spiked matrix after SP	MET: > 91; GMP: > 91; PIO: > 91
Shantikumar et al. [34]	LIN, SAX,SIT, TEN and VDP	UPLC–MS/MS	LLOQ: LIN and TEN = 0.1, and SAX, SIT and VDP = 0.5	LIN and TEN = 1–1,000; SAX, SIT and VDP = 0.5–1,000 ng mL^{-1}	Spiked matrix before SP × solution	LIN: 91.67; SAX: 94.32; SIT: 95.37; TEN: 93.29; VDP: 96.82
Sorensen, 2011 [35]	BUF, MET and PHE	HPLC–MS/MS	LOD/LLOQ: BUF = 6/25, MET = 10/25 and PHE = 11/25	BUF = 0.025–2.4, MET = 0.025–12 and PHE = 0.025–0.6 μg mL^{-1}	Spiked matrix before SP × spiked matrix after SP	BUF: 93; MET: 91; PHE: 89
Tirumala, Lakshmi, 2009 [36]	GCZ, GMP, GPZ, PIO, REP and ROS	HPLC (UV)	LLOQ: GCZ, GMP, GPZ, PIO, REP and ROS = 0.1	GCZ, GMP, GPZ, PIO, REP and ROS = 0.1–100 μg mL^{-1}	Not performed	Not performed
Viana et al. [37]	CHL, GCZ and GMP	HPLC (UV)	LLOQ: CHL and GCZ = 1000; and GMP = 100	CHL = 1–50, GCZ = 1–10, and GMP = 0.1–1 μg mL^{-1}	Spiked matrix before SP × spiked matrix after SP	CHL: 37.2–46.7; GMP: 49.3–71.9; GPZ: 50.2–59.4
Yardimci et al. [38]	MET and ROS	HPLC (UV)	LOD/LLOQ: MET = 100/250 and ROS = 50/100	MET = 0.25–2.5 and ROS = 0.1–2.5 μg mL^{-1}	Spiked matrix before SP × solution	MET: 91.48–97.62; ROS: 91.48–97.62
Zhang et al. [39]	MET and ROS	HPLC–MS/MS	LOD/LLOQ: MET = 1/5 and ROS = 1/1.5	MET = 5–3,000 and ROS = 1.5–500 ng mL^{-1}	Spiked matrix before SP × spiked matrix after SP	MET: 77.9–87.4; ROS: 80.2–83.6
Zhong et al. [40]	GCZ and MET	HPLC–MS/MS	LLOQ: GCZ = 10 and MET = 7.8	GCZ = 10–10,000 and MET = 7.8–4,678 ng mL^{-1}	Spiked matrix before SP × solution	GCZ: 88.1–104.0; MET: 71.0–83.6

Data: BUF, buformin; CAR, carbutamide; CHL, chlorpropamide; GBC, Glibenclamide; GBN, glibornuride;, GCZ, gliclazide; GMP, glimepiride; GPZ, glipizide; GQD, gliquidone; GSX, glisoxepide;, LIN, linagliptin; MET, metformin; MIG, miglitol; NAT, nateglinide; PHE, phenformin; PIO, pioglitazone; REP, repaglinide; ROS, rosiglitazone; SAX, saxagliptin; SIT, sitagliptin; TEN, tenegliptin; TZD, tolazamide; TBT, tolbutamide;, VDP, vildagliptin; UV, ultraviolet; HPLC, high performance liquid chromatography; UPLC, ultra performance liquid chromatography; SFC, supercritical fluid chromatography; MS/MS, tandem mass spectrometry; MS, mass spectrometry; ESI, electrospray ionization; APCI, atmospheric-pressure chemical ionization; CE, capillary electrophoresis; DAD, diode array detector; LOD, limit of detection; LLOQ, lower limit of quantification.

[a] Recovery values are presented as single or range values, depending on how reported in the record.

Bioanalytical Method Validation" by US FDA [3] (16 records) [9,12,15,20–23,27,29,30,32–34,38–40], "Harmonized Tripartite Guideline" by ICH [73] (four records) [16,17,36,38], "RDC 27/2012 – Minimum Requirements for Validation of Bioanalytical Methods" by ANVISA [5] (one record) [37], "The Fitness for Purpose of Analytical Methods" by Eurachem [98] (one record) [7], "Requirements for Initial Assay Validation and Publication in Journal of Chromatography B" by Wolfgang Lindner and Irving W. Wainer [99] (one record) [26], "Requirements for the Validation of Analytical Methods" by the Society of Toxicological and Forensic Chemistry [100] (one record) [18]; "Validation of New Methods" by Peters, Drummer and Musshoff, 2007 [101] (two records) [13,18] and "MS Identification Guidelines in Forensic Toxicology" by Australian/New Zealand Specialist Advisory Group in Toxicology [102] (one record) [13]. Although the EMA has a specific guideline for bioanalytical method validation, none of the records declared to have used it as guidance.

According to the US FDA, the main parameters used to demonstrate the acceptability of method performance and the reliability of the analytical results are selectivity, accuracy, precision, recovery, calibration curve, sensitivity/lower limit of quantification, and stability. In addition, ANVISA and EMA also add matrix effects and carryover to the list of fundamental validation parameters [3–5]. Besides these parameters, other optional parameters can be assessed, such as the limit of detection, robustness, and system suitability [3,73,103].

3.2.1. Selectivity

Selectivity is defined as the ability of a method to differentiate the analytes of interest and the internal standard (IS) from other compounds that may be present in a biological matrix, such as metabolites, drugs, impurities, decomposition products, or matrix components [3,104]. The guidelines indicate that selectivity should be evaluated in blank samples of the biological matrix obtained from at least six sources and ensured at the lower limit of quantification (LLOQ) [3–5].

ANVISA guideline establishes that when the matrix is plasma, four normal samples, one lipemic and one hemolyzed should be analyzed, and when the matrix is total blood, five normal samples and one lipemic samples should be tested. The importance of analyzing other matrices such as hemolyzed and lipemic plasma is to increase the robustness of the method that will be applied in routine analysis, where these kinds of samples are likely to occur (e.g. inappropriate sample collection and handling) [105,106].

In most articles, selectivity is generally demonstrated using images, through the comparison of a LLOQ chromatogram versus a blank sample chromatogram. However, the EMA and ANVISA guidelines also suggest a numeric value for evaluating this parameter: the response of interfering components near the analytes should be less than 20% of LLOQ and, when near the IS, 5% of its response maximum. Therefore, some articles may also present selectivity as a percentage value [4,5].

Of the 34 records included in this review, 29 reported that the method was selective [7–9,12–23,25–30,32–34,36–40] and 28 analyzed six sources or more matrix sources, as suggested by the guidelines [3–5]. Three of them also evaluated lipemic and hemolyzed samples [29,32,37]. Only three articles did not specify how this parameter was evaluated but claimed that the method was selective [21,25,36]. Almost all articles demonstrated selectivity in images; three reported it as numeric values [15,37,40]. Five articles did not evaluate the selectivity at all, and none of these records used a guideline for guidance [10,11,24,31,35]. The absence of selectivity can generate serious errors when not applied in routine analyses [107]. Other compounds present in the matrix could interfere with the analytical signal, resulting in an erroneous quantification.

3.2.2. Limit of detection and lower limit of quantification

The limit of detection (LOD) is the lowest amount of an analyte that can be detected with reliability but not quantified. The lower limit of quantitation (LLOQ) is defined as the lowest concentration of the analyte that can be quantitatively determined with acceptable accuracy and precision [3–5,101,108].

According to the US FDA and EMA guidelines, the analyte response at the LLOQ should be at least five times the blank response and correspond to the lowest calibration standard. The EMA guideline also states that "*LLOQ should be adapted to expected concentrations and to the aim of the study*." In other words, LLOQ does not necessarily need to be as low as it could if such low amounts are not expected in the sample. For example, chlorpropamide is an antidiabetic agent that shows plasma maximum concentration (C_{max}) values around 30 μg mL^{-1}, at a dose of 250 mg [37,109–111]. Taking into account the values from Letendre et al. for C_{max} (34 μg mL^{-1}), the elimination constant rate (0.0198 h^{-1}), and the usual dose interval of chlorpropamide, which is 24 h, the C_{min} would be not much less than about 21 μg mL^{-1}. Based on that, lower quantities in a TDM study are not expected, i.e. the calibration curve does not need to have a very low LLOQ. However, if the purpose of the method is to quantify minimal doses present in the sample, the LLOQ must be as low as possible, with accuracy and precision proved. Most of records considered LLOQ as 10 times the blank sample response.

None of the main guidelines describes how to evaluate LOD, nor do they require the assessment of this parameter. Despite this, 19 articles determined the LOD, considering it to be three times the blank plasma signal [7,9–11,13,16–19,24–26,29,31,32,35,38–40], as suggested in the ICH guideline (for analytical method validation) [73]. Although not mandatory, the determination of LOD is interesting and must be considered when the qualitative identification of the drug is important information. For example, in doping control analysis, the LOD is a very important tool. The presence of several substances even in non-quantifiable concentrations is relevant for the World Anti-Doping Agency (WADA) [112]. The LLOQ and LOD values of each method are presented in Table 1.

3.2.3. Calibration curve

The calibration curve represents the relationship between known amounts of the analyte in the sample and the response of the instrument. This relationship should be continuous and reproducible [3,113]. The guidelines recommend preparing the calibration curve in the same matrix in which the method will be applied later. Three calibration curves should be prepared with a minimum of six calibration levels (matrix sample processed with a fixed concentration of internal standard and different concentrations of the analytes), a blank sample (matrix without analyte and internal standard), and a zero sample (matrix without analyte but with internal standard); the last two kinds of samples are not used to calculate the calibration curve parameters. Additionally, the results should be analyzed by appropriate statistical methods, preferentially using the simplest model possible (linear model) [3–5].

Only 15 records followed the instructions regarding the number of curves [7,9,13,14,16–18,20,21,23,27,29,37,39,40], while 19 did not report how many curves were prepared [8,10–12,15,19,22,24–26,28,30–36,38]. Regarding the calibration levels, 24 records included six or more levels [7,9,11–23,25,27–30,33,34,37,39], as recommended. Five articles performed the curve with only four or five levels [8,24,26,31,35] and five did not provide the number of levels [10,32,36,38,40]. Moreover, only six studies reported including blank and zero samples in the preparation of the curve [11,21,29,37,39,40]. Sample number is very important to minimize the maximum error of the estimate. The larger the sample number, the smaller the error within a certain confidence interval [114]. In this way, it is critical to reduce the number of levels and curves, as it will directly affect the reliability of the method.

The curve range and the concentrations of the calibration levels should be chosen according to the expected real sample concentration, linearity, and LLOQ. The range cannot include values lower than LLOQ or higher than the upper limit of quantification (ULOQ) [3–5]. However, we noticed that, in the article by Jingar et al. [21], the first

point of the curve was inferior to the LLOQ, which may compromise the results, since this point was not tested for precision and accuracy. The range of the curve for each method is presented in Table 1.

The calibration levels are considered satisfactory when deviation is ≤15% of their nominal concentration, except for LLOQ, for which variation must be less than 20%. This specification should be met by at least 75% of the analyzed points [3–5]. However, only ten records followed this recommendation regarding curve specifications [15,17,21,27–29,33,37,39,40]. The other articles did not report whether these criteria were evaluated and considered the curve acceptable through the correlation coefficient (r). According to the literature [115–118], the correlation coefficient alone is not enough to assure linearity and the deviation should be evaluated during calibration curve validation. When the variations are greater than 15%, linearity cannot be guaranteed even if the correlation coefficient is > 0.99. To obtain a reliable regression, it is necessary to limit the deviation of the points [115–118].

3.2.4. Precision

Precision reflects the closeness between the values obtained through the repetitive performance of an experimental procedure, under specific conditions [73,104]. Precision is expressed as relative standard deviation (%RSD) or coefficient of variation (%CV), and it should be analyzed in a single analytical run (intra-batch precision) and between runs (inter-batch precision). The values should not exceed 15% for quality control (QC) samples, except for LLOQ, which should not exceed 20% [3–5].

The FDA suggests the evaluation of precision at three levels; the EMA recommends four levels (LLOQ, low, medium, and high QC samples) and ANVISA recommends five levels (LLOQ, low, medium, high, and dilution QC samples). In addition, the regulatory agencies recommend that precision should be demonstrated using a minimal of five determinations per concentration [3–5].

Of the 34 articles included in this review, 25 assessed precision according to the recommendations and obtained values within those established in the guidelines [7–9,12–17,19–23,27–29,31–34,37–40]. The other nine did not follow the guidelines or the values are out of the acceptable deviation [10,11,18,24–26,30,35,36]. Among these nine records, one performed this parameter in solution [25] and one article only mentioned that the method was precise but did not report how the parameter was evaluated and which values were obtained [36]. Three records presented values above the recommended level, with a CV higher than 20% for some points [13,18,26]. Five records did not report the number of replicates [16,18,19,23,36] and another five articles assessed this parameter at only one or two concentrations [11,24–26,35].

When this parameter is not correctly assessed, it means that the method does not have the minimal precision required, and is not suitable to quantify drugs in bioanalytical samples [3]. The miscalculated results obtained by these methods may lead to erroneous decisions, such as unnecessary dose adjustment, underdosing, and overdosing in evaluated patients.

3.2.5. Accuracy

Accuracy is defined as the closeness of the true value to the mean value obtained by the method. It is the deviation between these two values, measured as relative error (%RE). The deviation should not exceed 15%, except for the LLOQ, which should vary by less than 20% [3,4,104]. According to the main guidelines, accuracy should be determined within a single run (intra-day) and in different runs (inter-day), with a minimum of five samples per concentration [3–5]. The US FDA suggests a minimum of three concentration levels, the EMA recommends four (LLOQ, low, medium, and high QC) and ANVISA recommends five (LLOQ, low, medium, high, and dilution QC).

Twenty-four articles conducted this test following the guidelines [7–9,12,14–17,19–23,27–29,31–34,37–40]. Only three records did not assess inter-day accuracy [10,11,30], one did not assess this parameter [24], and one only mentioned that it was accurate, but did not mention how the test was performed and did not provide the results [36]. One record used two calibration levels [26] and two used one level [11,25]. These articles are in disagreement with the advice of the international regulatory agencies.

Two articles extrapolated the recommended deviation [13,18], which is worrisome in TDM, as it generates erroneous models in PK studies. For instance, when the method is put into practice, the real blood concentration can be higher (or lower) than the experimentally obtained value, leading to a dose adjustment that may expose the patient to a toxic drug concentration range (or to an ineffective drug concentration range). In the case of PK studies, the risk is related to incorrect dose calculations due to misleading PK parameters.

3.2.6. Recovery

Recovery measures the efficiency of the sample extraction process. It evaluates the ability of the sample preparation method in extracting the analyte from the biological matrix. This parameter is usually reported as a percentage value. The EMA and ANVISA guidelines do not establish specifications for the determination of recovery, but the US FDA recommends comparing the detector response of matrix samples spiked before sample preparation with standards in solution, which would represent 100% [3–5].

The ideal value for recovery is 100%, but smaller values are acceptable, as long as the recovery is precise, reproducible, and consistent [3]. For this reason, US FDA suggests that recovery should be determined using at least three levels, such as LQC, MQC, and HQC, with a maximum deviation of 15%.

Two articles presented deviation values superior to 15% [13,18]. Therefore, the recovery of these methods is not reproducible, which may compromise the results of other parameters such as precision and accuracy.

After the analysis of the 34 records, we noticed that 29 assessed this parameter [7–9,11–13,15–24,26–29,31–35,37–40]. Among these, 13 conducted this test comparing the matrix sample spiked before sample preparation with the standard in solution [15,17,19–21,23,26,28,31,32,34,38,40]. Another 13 records compared a matrix sample spiked before sample preparation with a matrix sample spiked after sample preparation [9,11–13,16,18,22,27,29,33,35,37,39], which may be a valid alternative to the US FDA guidelines, and is recommended by the Japanese document “Guideline on Bionalyticial Method Validation in Pharmaceutical Development” [119]. In both cases, the recovery is well-represented. When comparing a sample against the analyte in solution, it represents the recovery and also includes matrix interference. The other way is to compare a sample against the analyte in the matrix, fortified after extraction, reflecting the efficiency of extraction and the recovery itself [120]. Three records did not report how recovery was evaluated [7,8,24]. The recovery rates of each method as well as the sample preparation method are presented in Table 1.

3.2.7. Matrix effect

Matrix effect (ME) is the influence of other compounds present in the matrix on the response of the analytes [121]. It represents, along with selectivity, the main reason why a method cannot be developed and validated in solution and later applied to a different matrix such as plasma or serum. The complex components of the biological matrix are not present when validation in solution is performed, thus their influence would be only noticed through a lack of accuracy during method application.

The EMA and ANVISA documents describe quantitative analyses of ME, and the EMA guideline reinforces the notion that ME should be investigated when using mass spectrometric methods, and at least six lots of blank matrix (not pooled) should be tested. The ANVISA guideline mentions that if the matrix is plasma, eight distinct samples

should be analyzed, of which two should be lipemic and two hemolyzed, and when dealing with total blood, six samples should be evaluated, including two lipemic samples. For each sample, the signals of the analytes and IS in matrix should be divided by the signal in solution, obtaining the matrix factor (MF). The CV of the MF calculated should not be greater than 15%. It is recommended to perform this assay at low and high sample concentrations [4,5].

The importance of this parameter is related to the selectivity of the method; the more selective the technique, more attention should be given to ME. In the case of less selective techniques, such as photometry, the interfering components usually appear as visible peaks. On the other hand, in a more selective technique such as LC–MS, the interfering substances may not be visible in the monitored *m/z* ratio and may cause suppression or enhancement of the signal, a change in the baseline, contribute to chromatographic tailing, or have an impact on analyte retention time [122]. ME occurs especially when using electrospray ionization (ESI) as the source in mass spectrometry methods, because the ME phenomenon is related to the process of charging and desolvating the analytes in the liquid phase into gas ions introduced into the MS analyzer [122–124]. However, when using an APCI source, the analytes are charged in the gas phase, eliminating droplet generation or desolvation problems. Hence, ME is reduced, but not fully eliminated, since all of its causes are not yet completely understood [122]. Of the 24 methods using mass spectrometry included in this review, only four did not perform ME evaluation [14,15,26,34], but two of them used APCI as the ionization source.

Twelve records were removed from this review because validation was performed in solution with the aim of applying the method to blood samples [41–52]. An example of this problem can be found in one of the included articles, from Magni et al. [24]. The authors reported on the preparation of two different calibration curves, one in solution and another in a biological matrix. As result, they observed that the slope of the calibration curve for one of the analytes (chlorpropamide) in solution was about twice of that obtained in serum, which is most likely related to ME.

Two articles did not follow the main guidelines (that properly explain how to evaluate ME) and hence evaluated this parameter inadequately [9,24]. They compared blank matrix spiked with analytes before sample preparation with a pure solution of the analyte. If the analytes are added before sample preparation, the efficiency of the recovery will be considered in the evaluation, cloaking the real ME and giving the impression of a higher ME. In fact, the approach used by these authors is the procedure used to perform the recovery test [3].

A higher than recommended MF was observed in three records. Gonzalez et al. [16] and Di Rago et al. [13] developed methods for the simultaneous quantification of several drugs, not limited only to antidiabetic agents, in which 55 and 132 analytes were analyzed, respectively. When such a high number of analytes is quantified in a single method, it is almost impossible to escape the ME. Despite the authors' concern about the inevitable problem and efforts in optimizing sample preparation method, not all analytes presented a good response. Meanwhile, Binz et al. [11], who quantified only five antidiabetic agents in serum, obtained considerable high MF values (up to 56% of deviation) and, although these results were reported in the article, nothing was done about it or commented on. Since Binz et al. did not follow any guideline, the authors were probably not aware about MF limit values, reinforcing the importance of following a guideline for appropriate method development and validation.

3.2.8. Carryover

Carryover is the effect generated by the appearance of or increase in the analyte or internal standard signal after injection, caused by the residue of these substances from samples analyzed in the previous run. This problem can affect accuracy and precision, mainly in lower concentration samples [125]. Carryover should be assessed during method development to assure its elimination or minimization. The US FDA only mentions the test in its guideline, but the EMA and ANVISA include more detailed information. Carryover consists of the analysis of blank samples after the injection of a high concentration sample, and the response of the analyte in the blank sample is then compared with the response at LLOQ. The acceptance criterion is until 20% of the analyte response at the LLOQ and until 5% the response of the IS at the LLOQ. Regarding the 34 records included in the review, only six performed this test [19,23,28,29,34,37], despite its importance in influencing the results of other parameters such as precision and accuracy [125].

3.2.9. Stability

The instability of analytes in biological samples is associated with their physicochemical properties, concomitant medications, matrix components, and conditions of storage and analysis to which the samples are submitted; it is a source of over- or underestimation of the concentration of the analyte. To avoid this potential error, the behavior of the substances of interest should be monitored in solution and in a biological matrix. When evaluating the stability of the method, it is important to ensure that the concentration of the analytes in the sample will remain stable during the entire analysis and also to determine the ideal storage conditions to be used [126].

The stability study should simulate or reproduce the conditions likely to be encountered during the study, i.e. sample handling, analysis, and storage. For this, the following tests should be performed: the stability of analytes and IS in the matrix, the stability of analytes and IS in stock solutions, and the stability of the analytes and IS in working solutions [113,126].

The stability of analytes and IS in the matrix should be assessed with at least three replicates at low and high QC samples and compared to freshly prepared calibration curves. The result is considered acceptable when the deviation is less than 15% from the theoretical concentration. The stability of the matrix is evaluated as bench-top (time and conditions to which the samples are submitted during sample handling in laboratory), long-term (time and conditions to which the samples are submitted during the whole study period), and processed sample/autosampler (time and conditions to which the samples are submitted during a whole batch analysis) stabilities and through freeze thaw cycles [3–5,113].

The stability of stock solutions and working solutions should be evaluated at room temperature within a short period of time and under storage conditions for longer periods, with a subsequent comparison with freshly prepared samples. The deviation should not exceed 10% [3–5].

Among the included articles, we observed that 12 did not conduct an evaluation of freeze/thaw cycle stability [7,10,11,16–19,23–26,35], 11 did not test bench-top stability [7,10,11,14,16,17,19,24–26], 11 did not test long-term stability [10,11,14,16–19,21,23–25], and 19 did not assess processed sample stability [7–11,13,16–19,23–26,30,31,35,36,38]. Furthermore, 25 articles did not test analyte stability in solution [7–11,13–16,18–22,24–26,30,31,33–36,38,40].

These results demonstrate a serious problem with the validated methods. According to this, there is no evidence that these methods could be successfully applied in routine analysis. The plasma matrix is a complex sample, with many enzymes and metabolites, which may develop a significant interference after some time and/or under some conditions [127,128]. Only three articles performed a complete stability validation, according to the guidelines. All other methods are not suitable for the quantification of real samples because there is no guarantee that the parameter remains the same over time [3].

3.2.10. Robustness

The definition of robustness, sometimes called ruggedness, is not present in the three main guidelines for bioanalytical method validation (EMA, US FDA, and ANVISA) [3–5]. Although not requiring the evaluation of robustness, all three guidelines explain that when minor

changes are made in the method, partial validation is necessary. The ICH guideline [73] or ANVISA RDC 899 from 2003 [103], despite being assigned to analytical methods validation, can be consulted for the assessment of this parameter. ICH defines robustness as the capacity of a method "*to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage*" and suggests which modifications should be experimented on. It provides important information and is strongly recommended when the intent is to reproduce and apply the same method in other studies. Furthermore, interactions among factors may occur, and it is important to point out the critical points and respect the pre-determined conditions [122,129]. For liquid chromatographic methods, variations in mobile phase pH, mobile phase composition, different columns (different lots and/or suppliers), temperature, and flow rate can be tested [73]. When using mass spectrometry, variations in the drying gas temperature, nebulizer gas pressure and flow rate, ion source configuration (nebulizer position), and ion source conditions (nebulizer aging, ion source contamination) can be also assessed [130].

Three articles included in this review conducted robustness assays. Rashid et al. [31] tested different HPLC apparatuses; Tirumala et al. [36] evaluated modifications in the composition of the mobile phase and flow rate, and Yardimci *et al.* [38] modified the pH of the buffer and its concentration. In these articles, a one-variable-at-a-time procedure was performed. In this procedure, the critical points of the method can be identified. Usually, the results are reported as RSD (%) [31,36,129,130], but they can be alternatively presented as the Student's *t*-test or ANOVA, to guarantee the statistical significance of the results [38,129,130].

3.2.11. System suitability

System suitability tests are proposed only by the US FDA, which defines these as the "*determination of instrument performance by analysis of a set of reference standards conducted prior to the analytical run*" [3]. However, since system suitability is not classified as a fundamental validation parameter, but rather as an expectation of a good system to use a validated bioanalytical method, it is usually not performed.

The US FDA guideline does not clarify which parameters should be assessed in system suitability, only stating, "*apparatus conditioning and instrument performance should be determined using spiked samples independent of the study calibrators, QCs, or study samples*". System suitability is better explained in the USP Pharmacopeia [131], which considers that these "*tests are based on the concept that the equipment, electronics, analytical operations, and samples analyzed constitute an integral system that can be evaluated as such*". The recommendation is to assess peak resolution (Rs), the number of theoretical plates of the column (N), and the separation (α), capacity (k), and symmetry (As) factors [131]. Once these parameters are verified, the equipment is considered satisfactory and the analytical run can be carried out.

Only one article [38] executed system suitability tests. The authors evaluated the capacity factor and symmetry of the peaks.

3.3. Limitations of the methods

Several problems were found in the development of the bioanalytical methods included in this review. In fact, the majority were developed for a few drugs and therapeutic classes; therefore, they are not applicable for most of the combinations recommended for type 2 diabetes treatment [132–134].

Frequent discrepancies among the articles regarding the execution of the validation parameters were observed. This inconsistency may be related to the choice of the guideline: some records followed analytical validation guidelines while others did not follow any guideline at all. Besides, in some of the records that followed appropriate bioanalytical guidelines, the execution was not correct, according to the recommendation. The main validation problems were: (1) not assessing the parameter or not describing how it was assessed in the article, (2) extrapolation of the maximum variation allowed for the parameter, (3) inappropriate sampling number, and (4) not performing inter-day assays. Considering the importance of appropriate method validation, these problems may diminish the confidence and suitability of the method regarding its intended use.

Although bioanalytical method validation parameters have been comprehensively discussed in the literature [2,108,121,122,124,130,135–140], the publication of incorrectly validated methods can still be observed. Therefore, this review aimed to draw attention to this problem, by exemplifying it and pointing out the many problems that may be caused by incorrect validation. It is necessary to determine what is possibly occurring. These inappropriately validated methods are available in the literature, and may not actually be suitable for the intended purpose. In this review, all the records, except for the one from Magni et al. [24], were published after 2001, the year of release of FDA guideline for bioanalytical method validation. And, as shown throughout this review, many records did not follow the orientation guideline and/or assessed the parameters inappropriately. Most of the articles did not fully discuss validation parameter results and procedures, which is necessary to demonstrate the reliability of the method and therefore of all study results.

However, some articles performed the validation steps according to the guidelines [12,20–23,27–29,33,37,39,40]; these studies can be used as a model and should be consulted by other researchers.

4. Conclusion

This systematic review was able to gather all records present in the scientific literature about validated bioanalytical methods for the quantitation of oral antidiabetic agent associations in human blood. A highly alarming variation in the validation procedure was observed in recent articles. The improper assessment of the analytical parameters required for validation can lead to discrepant results between theoretical and practical concentrations, generating an excessive number of unreliable results, despite of all the new technologies currently available. Furthermore, the methods reported in many articles were not validated according to the specific guidelines, easily accessible through the internet and free for consultation. In bioanalytical analysis, several interfering factors can be present, which must be taken into consideration in order to achieve a reliable and truthful method. This review covered the main problems present in bioanalytical validation and raises the alarm regarding the need for standardization, as well as stronger commitment and responsibility among the analytical laboratories.

Conflicts of interest

The authors declare no conflicts of interest.

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