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Determination and validation of tiaprofenic acid in human plasma: A detailed LC-MS/MS-based analysis following ICH M10 guidelines and the accuracy profile approach

Houda Bouchafra[a,b*], Aimen El Orche[a,b], Choukri El Khabbaz[b,c], Amine Cheikh[b,d], Miloud El Karbane[b,f], My El Abbes Faouzi[b,e], Yahia Cherrah[b,e], Lhoussaine Zarayby[a] and Aziz Hirri[a]

[a]*Laboratory of drug sciences, biomedical and biotechnology research. Faculty of Medicine and Pharmacy, Hassan II University of Casablanca, Morocco*
[b]*Sheikh Zaid Center for Bioequivalence Studies, Rabat, Morrocco*
[c]*Laboratory of Materials Nanotechnologies and Environment, Faculty of Science, Mohammed V University, Rabat, Morocco*
[d]*Department of Pharmacy, Faculty of Pharmacy, Abulcasis University, Rabat, Morocco*
[e]*Laboratory of Pharmacology and Toxicology, Faculty of Medicine and Pharmacy, Mohammed V University, Rabat, Morocco*
[f]*Laboratory of Analytical Chemistry & Bromatology, Faculty of Medicine and Pharmacy, Mohammed V University in Rabat, Rabat, Morocco*

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A B S T R A C T

The validation of bioanalytical methods holds critical importance for regulatory agencies and organizations dedicated to ensuring the safety, efficacy, and quality of pharmaceuticals. In this context, the recent release of the ICH M10 guideline in May 2022 represents a significant milestone in standardizing bioanalytical method validation globally. However, this guideline lacks explicit experimental protocols for implementation. In this study, we address the practical implementation of the newly released ICH M10 guideline by providing a detailed validation protocol for a bioanalytical method. Our method specifically targets tiaprofenic acid, a widely used nonsteroidal anti-inflammatory drug. Tiaprofenic acid is a critical component in bioequivalence studies, underscoring the necessity for precise and accurate quantification within complex biological matrices. The integration of the accuracy profile approach, a statistical tool, enhances the significance of this work. This approach aids in assessing the accuracy and precision of bioanalytical methods, establishing confidence intervals around measured concentrations, and quantifying the level of accuracy and precision expected when using the validated method.

1. Introduction

Bioanalysis has always been a subject of paramount interest to numerous regulatory agencies and organizations committed to safeguarding public health by ensuring the quality, efficacy, and safety of pharmaceutical drugs [1]. Among these regulatory bodies, the United States Food and Drug Administration (FDA) emerged as one of the pioneers in developing guidelines for the validation of bioanalytical methods. Over time, these guidelines have evolved in response to the dynamic landscape of bioanalysis [2]. Concurrently, the European Medicines Agency (EMA) has formulated its own guide on bioanalytical method validation, albeit with certain nuances compared to the FDA's standards [3].

A significant milestone in the realm of bioanalytical method validation was reached in May 2022 when the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) released the latest iteration of the ICH-M10 guideline. This guideline represents a significant stride towards standardizing the validation of bioanalytical methods on a global scale [3]. While the ICH-M10 guideline meticulously outlines the parameters for validating bioanalytical methods and establishes acceptance criteria, it does not provide explicit experimental protocols for their

* Corresponding author
E-mail address houdabouchafra@gmail.com (H. Bouchafra)

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application. In response to this need, this work aims to fill the gap by presenting a comprehensive experimental protocol for validating a bioanalytical method in accordance with the ICH-M10 standard.

For our experimental validation, we have selected tiaprofenic acid as the target analyte. Tiaprofenic acid, a nonsteroidal anti-inflammatory drug (NSAID) belonging to the propionic acid group, derived from benzoic acid, is widely used as an analgesic and antipyretic agent [4]. Its inclusion in bioequivalence studies underscores the importance of accurate quantification in biological matrices, as this is pivotal for making critical determinations regarding drug safety and efficacy [5]. In the context of bioequivalence studies, the precise measurement of pharmaceutical dosage in biological matrices assumes paramount significance [1-6]. Consequently, the development and validation of bioanalytical methods are imperative to establish the credibility and robustness of study outcomes [7, 8].

The amounts of tiaprofenic acid in different matrices can be determined using a number of techniques that have been documented, such as high-performance liquid chromatography (HPLC) [8–12], HPLC-MS-MS [13], and HILIC-MS/MS [14]. Most of these techniques employ extraction methods, like liquid–liquid extraction (LLE) [8, 10, 12], solid-phase extraction (SPE)[10] or protein precipitation [11, 12, 14].

In the present study, a new, sensitive, specific, and rapid LC-MS/MS method was established for the analysis of tiaprofenic acid in human plasma with protein precipitation. Compared to liquid-liquid or solid-liquid extraction, the assay uses less solvent and offers a quick pretreatment step. It is also straightforward and does not require steps for solvent evaporation reconstitution [8, 10, 12]. Furthermore, it has high sensitivity with lower LLOQ concentrations than the existing methods [8, 10, 14]. The method was successfully validated according to ICHM10.

The ICH-M10 guideline serves as a compass, offering recommendations and validation criteria for the rigorous evaluation of bioanalytical methods [3]. However, it is the integration of the accuracy profile approach that elevates the significance of this work. The accuracy profile approach, a statistical tool, plays a pivotal role in ensuring the precision and accuracy of bioanalytical methods [15, 16]. It provides a systematic means to assess the reliability of analytical results by establishing confidence intervals around the measured concentrations [17]. This approach helps quantify the degree of accuracy and precision that can be expected when using the validated method [12].

In summary, this research endeavor bridges the gap between the ICH-M10 guideline and practical implementation, ultimately facilitating the development of validated bioanalytical methods essential for conducting rigorous bioequivalence studies and ensuring the safety and efficacy of pharmaceutical interventions. The incorporation of the accuracy profile approach underscores our commitment to delivering not just validated methods but also robust and reliable tools for drug evaluation in the pursuit of better public health and patient outcomes.

2. Materials and methods

2.1 Chemical Reagents and Equipment

Tiaprofenic acid was procured from Erregierre (San Paolo d'Argon, Italy), while the internal standard (IS), Ibuprofen, was sourced from Sigma Aldrich (Saint Louis, USA). Acetonitrile (LC/MS grade and HPLC grade) was acquired from VWR (Fontenay-Sous-Bois, France), while methanol (LC/MS grade and HPLC grade) was obtained from Merck (Darmstadt, Germany). Formic acid (ACS grade) was purchased from Scharlau (Barcelona, Spain), and ammonium formate was procured from HIMEDIA (India). Ultrapure water, with a resistance of >18.0 Ω/cm, was employed.

Drug-free fresh human plasma, collected from healthy subjects with lithium heparin as an anticoagulant, was sourced from BD-Vacutainer (UK) and stored at freezing temperature (~-25°C) until required.

2.2 *LC-MS/MS Conditions*

The UHPLC system comprised an Agilent 1290 Infinity II quaternary pump (Germany), an Agilent 1290 Infinity II autosampler (Germany), and an Agilent 1290 Infinity II column thermostat (Germany). Chromatography was conducted on a Phenomenex C18 analytical column (150 mm × 2 mm, 4 µm particle size; Phenomenex, USA) at a flow rate of 0.2 mL/min. Mobile phases consisted of 2-mM ammonium formate in water with 0.1% formic acid (A) and acetonitrile (LC/MS grade) (B). Optimal chromatographic separation was achieved with a 50% A and 50% B composition. The column temperature was maintained at 40°C, and tiaprofenic acid and ibuprofen (IS) eluted at approximately 4.87 and 7.12 minutes, respectively.

Mass spectrometric analysis was performed using an Agilent 6420 triple quadrupole mass spectrometer (Agilent, Germany) controlled by MassHunter B.09.00 software. The mass spectrometer operated in the positive Multiple Reaction Monitoring (MRM) mode with a dwell time of 200 ms per transition and utilized Electrospray Ionization (ESI). MRM transitions were 261 >> 105 and 183 for tiaprofenic acid and 207 >> 161 for IS, ensuring the widest resolution for all analytes. Fragmenter settings were optimized at 130 V for tiaprofenic acid and 132 V for IS, with collision energy set at 18 eV for tiaprofenic acid and 10 eV for IS.

2.3 Preparation of Calibration Standards and Quality Control Samples

Calibration samples were prepared by mixing human plasma (475 µL) with an aliquot (25 µL) of Tiaprofenic acid stock solutions in methanol (2, 10, 20, 100, 200, 250, 500, and 1000 ng/µL) to achieve nominal concentrations of 100, 500, 1000, 5000, 10000, 12500, 25000, and 50000 ng/mL for tiaprofenic acid. Quality control (QC) samples were prepared by mixing human plasma (475 mL) with an aliquot (25 µL) of tiaprofenic acid stock solutions in methanol (2, 4, 400, 800 ng/µL) to provide LLOQ, low, medium, and high QC samples with nominal concentrations of 100, 200, 20000, and 40000 ng/mL for tiaprofenic acid. Sample Preparation: Sample preparation involved a protein precipitation process. Specifically, 25 µL of IS stock solution in methanol (500 ng/µL) was added to 500 µL of plasma containing tiaprofenic acid. After agitation, 500 µL of methanol and 1000 µL of acetonitrile were introduced, followed by 30 seconds of agitation. The samples were then centrifuged for 10 minutes at 5000 rpm. The extracted samples were filtered and diluted by half with A (2 mM ammonium format with 0.1% formic acid). Finally, an aliquot (5 µl) was injected into the UHPLC system.

2.4 Bioanalytical Method Validation

The work conducted in this study adhered rigorously to the principles of Good Laboratory Practice, as stipulated by the Organisation for Economic Cooperation and Development (OECD). Additionally, all validation experiments were executed in accordance with the ICH M10 guideline on Bioanalytical Method Validation and Study Sample Analysis [3]. However, it is noteworthy that the statistical evaluation of accuracy and precision of the method diverged from the ICH M10 guideline and instead followed the guidelines set forth by the SFSTP [13].

The plasma used in the validation study was human plasma obtained by centrifuging blood collected in heparin tubes from healthy donors. This process was conducted following approval from the ethics committee of the Sheikh Zaid Foundation in Rabat, Morocco.

2.4.1 Selectivity

Selectivity, as defined by ICH M10, signifies the capacity of an analytical method to differentiate and quantify the target analyte amidst potential interfering substances in the blank biological matrix. In this context, chromatograms were generated for blank plasma obtained from six individual sources, blank plasma spiked with tiaprofenic acid at the Lower Limit of Quantification (LLOQ) concentration, and the Internal Standard (IS) at the concentration utilized in the study.

2.4.2 Specificity

Specificity, as per ICH M10, denotes the ability of a bioanalytical method to detect and distinguish the target analyte from other substances, including its related substances. In this vein, chromatograms were acquired for blank plasma, blank plasma spiked with tiaprofenic acid at the LLOQ concentration, and blank plasma spiked with IS at the concentration employed in the study.

2.4.3 Matrix Effect, Extraction Recovery & Process Efficiency

Evaluation of the matrix effect encompassed an analysis of low, medium, and high Calibration Quality Control samples (CQs) (n=4 for each level) prepared in the mobile phase (designated as A), those same CQ levels prepared in blank plasma before extraction from six different individual sources (designated as B), and those same CQ levels prepared in extracted blank plasma from six different individual sources (designated as C). Three parameters were scrutinized:

Matrix Effect (ME) on the compound's LC-MS/MS response: ME = (average of peak areas (C) / average of peak areas (A)) × 100.

Extraction Recovery (ER), accounting for the matrix effect: ER = (mean of peak areas (B) / mean of peak areas (A)) × 100.

Process Efficiency (PE), representing the extraction yield of the sample preparation method, disregarding the matrix effect: PE = (mean of peak areas (B) / mean of peak areas (C)) × 100.

Any ratio falling below 85% or exceeding 115% indicates the presence of an endogenous matrix effect.

2.4.4 Calibration and Response Function

The LC-MS/MS system employed a variety of regression models chosen based on the calibration range's accuracy and the quality controls in use. The calibration curve was established using eight concentration levels, and the evaluation was based on three independent runs conducted on different days.

2.4.5 Accuracy and Precision

Accuracy and precision were assessed using four concentration levels as Quality Controls (QCs) within the calibration curve range, including the Lower Limit of Quantification (LLOQ), low, medium, and high QCs. These assessments were conducted both within-run and between-run using the same dataset. Within-run accuracy and precision were determined

using six replicate samples for each of the four QC levels. Between-run accuracy and precision were derived from five independent runs on different days.

The acceptance criterion for accuracy at each concentration level was set within ±15% of the nominal concentration, except at the LLOQ, where it was ±20%. For precision (%CV), the criterion was that it should not exceed 15%, except at the LLOQ, where it was ±20%. Compliance with this criterion necessitated that at least 2/3 of the total QCs and at least 50% at each concentration level met these requirements.

To statistically demonstrate the accuracy and precision of the method, an accuracy profile approach was applied. This approach utilizes tolerance intervals to calculate upper and lower limits at each concentration level, resulting in a visually comprehensible graph that aids in the assessment of method performance.

2.4.6 Stability

The study encompassed an investigation into the stability of tiaprofenic acid under various storage conditions using low and high-quality control concentrations.

2.4.7 Hemolysis Effect

The hemolysis effect test was conducted on hemolyzed plasma loaded with low and high-quality controls in triplicate. The mean accuracy of QCs was expected to fall within ±15% of the nominal concentration, with the precision (%CV) not exceeding 15%.

2.4.8 Dilution Integrity Test

The dilution integrity test was carried out at twice the concentration of the high-quality control, with five replicates. Here, the mean accuracy of the dilution QCs was required to be within ±15% of the nominal concentration, and the precision (%CV) should not surpass 15%.

2.4.9 Carryover Test

To evaluate the auto sampler's carryover, a specific sequence of injections was followed, including blank plasma, Lower Limit of Quantification (LLOQ), Upper Limit of Quantification (ULOQ), and blank plasma. The criterion for carryover in the blank plasma following the ULOQ was that it should not exceed 20% of the analyte response at the LLOQ and 5% of the response for the IS.

2.4.10 Accuracy Profile Approach

To robustly demonstrate the accuracy and precision of the method, an accuracy profile approach was applied. This approach utilizes tolerance intervals to calculate upper and lower limits at each concentration level, resulting in a visually comprehensible graph that aids in the assessment of method performance. The accuracy profile is a powerful tool based on total error, providing a guarantee and prediction of future results when employing an approved approach.

3. Results and discussion

3.1 Selectivity

The selectivity of the method was assessed by examining the absence of any interfering peaks that co-eluted with tiaprofenic acid and the Internal Standard (IS) at their respective retention times. It was observed that batches 1 and 2 of the blank plasma had higher interference compared to the others, possibly due to the presence of endogenous products in the plasma. However, the interference percentages for tiaprofenic acid at the Lower Limit of Quantification (LLOQ) were below 20% for all six batches of blank plasma, confirming the method's selectivity. Similarly, the interference percentages for the IS remained below 5%. These results demonstrate (Table 1) that the method is selective for tiaprofenic acid and the IS, as no significant interference was observed, confirming the reliability of the method for quantitative analysis.

Table 1. Selectivity result

Human plasma	Batch-1	Batch -2	Batch -3	Batch -4	Batch -5	Batch -6
Interference percentage (%) of tiaprofenic acid	7.38	3.79	1.50	0.98	0.77	0.72
Interference percentage (%) of IS	0.02	0.04	0.02	0.01	0.17	0.01

3.2 Specificity

The specificity of our method was rigorously evaluated through a meticulous examination of chromatograms representing different scenarios. **Fig. 1** displays these chromatograms, which include those for blank plasma, blank plasma spiked with tiaprofenic acid at the Lower Limit of Quantification (LLOQ) concentration, and blank plasma spiked with the Internal Standard (IS) at the concentration utilized in this study.

The key criterion for assessing specificity was the determination of retention times, which were found to be approximately 5 minutes for tiaprofenic acid and 7.4 minutes for IS. Significantly, the examination of these chromatograms revealed the complete absence of any interfering peaks in the blank plasma at the specific retention times corresponding to tiaprofenic acid and IS. This unequivocal absence of interference reinforces the method's exceptional specificity, affirming its suitability for precise and accurate analytical purposes.

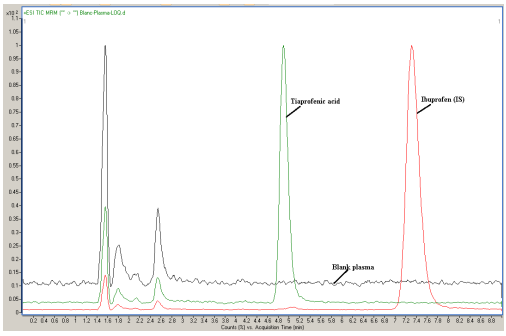

Fig. 1. Typical chromatograms obtained from tiaprofenic acid, ibuprofen (IS) and blank plasma.

3.3 Matrix effect

In order to comprehensively assess the matrix effect, extraction recovery, and process efficiency of tiaprofenic acid, we present the findings in **Table 2**. This analysis is crucial for understanding the impact of the biological matrix on the analytical method's performance. Remarkably, regardless of the concentration levels tested for tiaprofenic acid, no significant matrix effect was observed. The matrix effect (ME) values fell within the range of [90% - 109%], suggesting minimal interference from the matrix in the analysis. The process efficiency (PE) remained within the range of [87% - 116%], indicating the method's effectiveness in extracting the analyte from the matrix. Furthermore, the extraction recovery (ER) values obtained for both low and high concentrations of tiaprofenic acid were between [82% - 105%], affirming the method's reliability in recovering the analyte from the biological matrix.

Table 2. Matrix effect result

		Tiaprofenic acid					
			LQC (200 ng/ml)			HQC (40000ng/ml)	
		Tiprofenic acid preparing in mobile phase	Tiaprofenic Acid preparing in extracted blank plasma	Tiaprofenic acid preparing in blank plasma before extraction	Tiprofenic acid preparing in mobile phase	Tiaprofenic Acid preparing in extracted blank plasma	Tiaprofenic acid preparing in blank plasma before extraction
Plasma control-1	Area Mean	18145.01	18703.82	18934.87	6052813.13	5469410.50	5465336.64
	STD	1128.68	193.90	249.21	844062.99	10848.04	351220.47
	CV %	6.22	1.04	1.32	13.94	0.20	6.43
	ME		103.08			90.36	
	ER		104.35			90.29	
	PE		101.24			99.93	
Plasma control-2	Area Mean	18145.01	19359.94	18053.78	6052813.13	6465180.12	5689189.69
	Standard deviation	1128.68	160.03	250.29	844062.99	89070.50	118996.97
	CV %	6.22	0.83	1.39	13.94	1.38	2.09
	ME		106.70			106.81	
	ER		99.50			93.99	
	PE		93.25			88.00	
Plasma control-3	Area Mean	18145.01	19658.51	18567.70	6052813.13	6465180.12	6255489.38
	Standard deviation	1128.68	173.92	244.13	844062.99	89070.50	197894.89
	CV %	6.22	0.88	1.31	13.94	1.38	3.16
	ME		108.34			106.81	
	ER		102.33			103.35	
	PE		94.45			96.76	
Plasma control-4	Area Mean	18145.01	18289.31	17839.32	6052813.13	5469410.50	6368561.23
	Standard deviation	1128.68	141.66	251.53	844062.99	10848.04	129947.42
	CV %	6.22	0.77	1.41	13.94	0.20	2.04
	ME		100.80			90.36	
	ER		98.32			105.22	
	PE		97.54			116.44	
Plasma control-5	Area Mean	18145.01	19907.54	18573.06	6052813.13	5703717.02	5749054.35
	Standard deviation	1128.68	113.04	296.31	844062.99	29775.83	127751.15
	CV %	6.22	0.57	1.60	13.94	0.52	2.22
	ME		109.71			94.23	
	ER		102.36			94.98	
	PE		93.30			100.79	
Plasma control-6	Area Mean	18145.01	18572.06	19203.24	6052813.13	5693190.39	5023272.32
	Standard deviation	1128.68	270.37	210.87	844062.99	237057.05	132605.99
	CV %	6.22	1.46	1.10	13.94	4.16	2.64
	ME		102.35			94.06	
	ER		105.83			82.99	
	PE		103.40			88.23	

These findings collectively reinforce the robustness and reliability of our analytical method in the presence of diverse biological matrices, highlighting its suitability for accurately quantifying tiaprofenic acid.

3.4 Calibration and response function

Our calibration process for tiaprofenic acid meticulously evaluated various regression models to determine the most suitable model for calculating sample concentrations accurately. The assessment revealed that a quadratic model with a weighting of 1/concentration^2 provided the most robust and accurate results across the calibration curve. This model selection is crucial, as it directly impacts the accuracy and reliability of concentration calculations.

The nominal percentages of the three distinct calibration ranges are presented in Table 3. These ranges cover a wide spectrum of concentrations, ensuring the method's applicability across a broad analytical range. It is noteworthy that the accuracy of the back-calculated concentrations for each calibration standard falls comfortably within the stringent criterion of ±15%. This indicates the method's precision and reliability in accurately quantifying tiaprofenic acid concentrations, regardless of the specific concentration levels.

Table 3. Calibration results

		Nominal concentration (ng/mL)							
		100	500	1000	5000	10000	12500	25000	50000
Accuracy (%)	1	97.19	102.36	102.22	98.67	101.54	90.86	105.8	100.16
	2	97.95	100.56	100.14	100.8	100.18	97.72	100.72	104.51
	3	104.46	90.25	88.71	106.94	105.93	105.52	103.77	98.58

These findings collectively validate the robustness and accuracy of our calibration process and demonstrate the method's suitability for precise quantification of tiaprofenic acid concentrations across a wide range of values. This level of accuracy is essential for various bioanalytical applications where reliable concentration determination is paramount.

3.5 Accuracy and precision

The accuracy of our method was calculated by comparing the determined concentrations to the nominal values. Across all concentration levels, ranging from LLOQ to CQH, the accuracy consistently ranged from 80.9% to 109% of the nominal values. These accuracy percentages indicate that our method provides reliable and consistent measurements, as they are well within acceptable limits.

Precision, often expressed as the coefficient of variation (%CV), provides insights into the repeatability and reproducibility of the method. The precision values, expressed as %CV, ranged from 0.45% to 11.9%. These precision values are indicative of excellent repeatability and reproducibility, as they are substantially below the acceptable limit of 20% at the LLOQ and 15% at all other concentration levels.

The combined assessment of accuracy and precision underscores the robustness and reliability of our method for tiaprofenic acid quantification. Notably, at all concentration levels, the accuracy and precision values remained well within the predefined acceptance criteria. Specifically, accuracy values were <20% at the LLOQ and <15% at all other levels. Consequently, our method meets the stringent criteria for accuracy and precision, reinforcing its suitability for accurate tiaprofenic acid determination in diverse analytical applications.

Table 4. Accuracy and precision result

Analyte	Nominal Conc. (ng/mL)	Within-run (n=6)			Between-run (n=30)		
		Calculated Conc. (ng/mL) Mean±SD	Accuracy (%)	Precision	Calculated Conc. (ng/mL) Mean±SD	Accuracy (%)	Precision
Tiaprofenic acid	LLOQ (100 ng/mL)	94.8 ± 2.17	94	2.28	96.2 ± 11.5	96.2	11.9
	QCL(200 ng/mL)	207 ± 19.1	103	2.24	197 ± 14.8	98.3	7.51
	QCM(20000 ng/mL)	20000 ± 900	100	4.49	20600 ± 1490	103	7.26
	QCH(40000 ng/mL)	37300 ± 2500	93.3	6.69	40700 ± 2190	102	5.39

These results unequivocally establish the robustness and reliability of our method for tiaprofenic acid quantification. The method's consistent accuracy and precision, well within stringent acceptance criteria, demonstrates its suitability for precise and reliable tiaprofenic acid analysis across a wide range of concentrations. This level of analytical performance is fundamental to various bioanalytical applications where accuracy and precision are paramount.

The accuracy profile, depicted in **Fig. 2**, plays a critical role in assessing the method's reliability and its ability to consistently produce accurate results. This assessment incorporates tolerance intervals within the acceptance limits, emphasizing a crucial aspect of method validation.

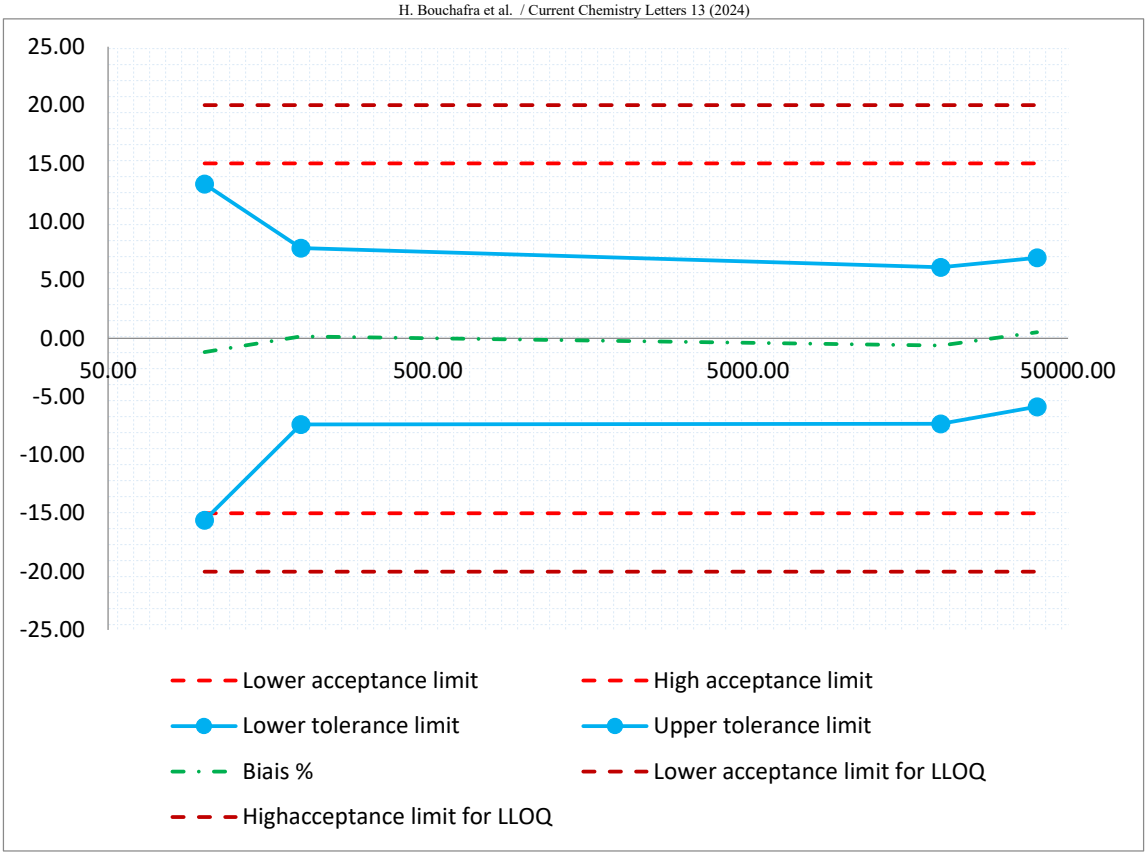

Fig. 2. Accuracy profil of tiaprofenic acid with λ= ±15%

The inclusion of tolerance intervals within the acceptance limits signifies a statistical assurance that the method can reliably deliver results meeting predefined criteria. Specifically, this means that the probability of the difference between the calculated concentrations and the reference values remaining below the acceptance limit exceeds the chosen β value, set as a proportion of future results at 90%.

In practical terms, this indicates that across a concentration range spanning from 100 ng/ml to 40000 ng/ml, the analyst can have confidence that the method consistently maintains an average β probability of producing results falling within the acceptable limits. This statistical assurance provides a high level of confidence in the method's ability to yield accurate and precise results consistently over a wide range of tiaprofenic acid concentrations.

This utilization of tolerance intervals and the β value underscores the method's robustness and its suitability for bioanalytical applications where maintaining consistent accuracy and precision is essential. It offers a valuable tool for quality control and assurance, enhancing the method's reliability and trustworthiness in various analytical settings.

3.6 Stability Assessment

Ensuring the stability of tiaprofenic acid under various conditions is integral to establishing the reliability and applicability of our method. The stability tests conducted under different scenarios yielded encouraging results, reaffirming the method's robustness.

3.6.1 Freeze-Thaw Stability

Tiaprofenic acid exhibited remarkable stability during three consecutive freeze-thaw cycles. The mean calculated concentrations for both low (QCL) and high (QCH) quality controls, as shown in **Table 5**, remained consistent, with accuracy percentages ranging from 101.6% to 111.7%. This outcome underscores the method's suitability for handling samples subject to multiple freeze-thaw cycles, a condition often encountered in real-world sample processing.

3.6.2 Bench-Top (Short-Term) Stability

Short-term benchtop stability, conducted over 8 hours at 22°C, demonstrated the resilience of tiaprofenic acid. The calculated concentrations for QCL and QCH quality controls remained stable, with accuracy percentages of 112.7% and 106.5%, respectively. This result indicates that the method is well-suited for short-term sample storage under ambient conditions without compromising accuracy.

3.6.3 Auto-Sampler Stability

The method also exhibited commendable stability during auto-sampler storage for an extended period of 72 hours at 10°C. The calculated concentrations for both QCL and QCH quality controls remained consistent, with accuracy percentages of 100.8% and 108.6%, respectively. This finding is essential for analytical workflows that involve automated sample handling and storage.

3.6.4 Long-Term Stability

Perhaps most impressively, tiaprofenic acid demonstrated exceptional long-term stability during a 164-day storage period at -25°C. The calculated concentrations for QCL and QCH quality controls remained virtually unchanged, with accuracy percentages of 114.5% and 105.11%, respectively. This remarkable stability over an extended duration highlights the method's reliability for long-term sample storage, a critical aspect of bioanalytical studies.

Table 5. stability result

Stability experiments	Mean calculated Conc. (n=3)		Accuracy (%)	
	QCL (200 ng/mL)	QCH (40000 ng/mL)	QCL (200 ng/mL)	QCH (40000 ng/mL)
Freeze-thaw matrix stability (3rd cycles)	203.2	44668	101.6	111.7
Bench top (short-term) matrix stability (8 h at 22°C)	225.5	42619	112.7	106.5
Auto-sampler matrix stability (72 h at 10 °C)	201.7	43463	100.8	108.6
Long-term matrix stability (164j at -25°C)	229.1	43585	114.5	105.11

3.7 Hemolysis Effect

The assessment of the hemolysis effect on tiaprofenic acid concentrations at both QCL and QCH using hemolyzed plasma produced encouraging results. The accuracy values obtained for each concentration were 97.2% and 99.7%, respectively, both well below the accepted threshold of 15%. This outcome firmly establishes the absence of a hemolysis effect, affirming the method's suitability for analyzing plasma samples that may be subject to hemolysis.

3.8 Dilution Integrity Test

The dilution integrity test, conducted on a plasma sample loaded with twice the concentration of CQH (40000 ng/mL), yielded accuracy and precision results of 96.08% and 8.21%, respectively. These results indicate that tiaprofenic acid samples can be reliably diluted by a factor of 2, extending the range of the calibration curve. This flexibility in sample dilution is valuable in situations where samples with high concentrations need to be analyzed accurately.

3.9 Auto-Sampler Carryover Test

To investigate the potential carryover effect of the auto-sampler, a sample of blank plasma was injected immediately after a sample from the upper limit of quantification of tiaprofenic acid. The interference percentages for tiaprofenic acid and IS were found to be 0.62% and 0.015%, respectively, both comfortably below the 20% threshold for tiaprofenic acid and 5% for IS. This outcome provides strong evidence of the absence of auto-sampler carryover for tiaprofenic acid and IS, ensuring the integrity of subsequent sample analyses.

4. Discussion

In this study, we developed and validated a method for quantifying tiaprofenic acid in human plasma which is essential for bioequivalence studies. LC-MS/MS analysis was chosen due to its superior specificity and selectivity compared to HPLC methods [8–12]. For sample preparation, we opted for protein precipitation over liquid-liquid extraction and SPE methods because of its simplicity, speed, and minimal solvent requirement, which reduces the risk of cross-contamination [8, 10, 12].

Our validation process followed the ICH M10 guideline, providing a comprehensive framework for bioanalytical method validation. This guideline offers distinct advantages over the FDA-2001 and ICH-Q2 guidelines, particularly in addressing the complexities of biological matrices [3].

We applied the accuracy profile approach, a representative statistical tool, to assess the precision and accuracy of our method comprehensively [20]. This approach allowed us to visualize our validation results effectively and ensured the reliability and robustness of our method, crucial for bioequivalence studies.

Our study aligns with the latest standards in bioanalytical science, emphasizing the importance of integrating modern statistical approaches like the accuracy profile. By upholding the highest standards of quality and reliability, our work

contributes to the advancement of bioanalytical science and underscores our commitment to ensuring the safety and efficacy of drug therapies.

In the dynamic landscape of pharmaceutical research and development, validated bioanalytical methods are indispensable. Moving forward, our validated method, supported by rigorous assessments like the accuracy profile, will play a vital role in safeguarding public health and advancing our understanding of drug therapies.

5. Conclusion

In this research, we have developed and validated a robust bioanalytical method for the quantification of tiaprofenic acid in human plasma using LC-MS/MS. Following the guidelines outlined in the ICH M10, we have thoroughly assessed the method's specificity, selectivity, accuracy, precision, and linearity.

Our study not only contributes to the field of bioanalytical science but also underscores the importance of adhering to the latest guidelines and employing modern statistical approaches such as the accuracy profile. By ensuring the reliability and accuracy of our method, we are better equipped to support bioequivalence studies and contribute to the advancement of drug therapies.

Moving forward, the validated method presented in this study can serve as a valuable tool for pharmacokinetic studies and therapeutic drug monitoring, ultimately improving patient care and advancing pharmaceutical research.

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