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Modified QuEChERS-LC-MS/MS Method for Forensic Quantification and Validation of Acyclovir and Amantadine from Biological Matrices

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Antiviral drugs exhibit various toxic effects such as teratogenicity, carcinogenicity and surprisingly neurotoxic effects that mimics that of drugs of abuse as indicated by the *in silico* toxicological studies performed and corroborated with published literature. Despite extensive studies on antiviral drugs, their forensic implications regarding individual toxicity, drug interactions, overdose and abuse have been overlooked. Consequently, there is a critical need to develop an analytical strategy for forensic detection of these antivirals. In this study, a simple and selective LC-MS/MS method is developed to determine toxic antivirals acyclovir and amantadine in urine, saliva and gastric fluid. The modified QuEChERS extraction was followed by chromatography on a C18 column with a gradient mobile phase of 5 mM ammonium formate and 0.1% formic acid in methanol. Detection was performed using a triple quadrupole tandem mass spectrometer. The validation of method, adhering to SWGTOX guidelines, demonstrated excellent linearity, precision and accuracy, covering a linear range of 5-200 ng/mL for both drugs. The limit of detection ranged from 14 to 20 ng/mL and the limit of quantification ranged from 40 to 63 ng/mL for acyclovir and amantadine. Finally, the validated method was successfully applied to the forensic detection of antiviral drugs in gastric lavage, exhibiting robustness and reproducibility.

Keywords: Antivirals, Acyclovir, Amantadine, QuEChERS, LCMS/MS, Forensic toxicology.

INTRODUCTION

Antiviral drugs are widely consumed across the globe as they play a crucial role in the treatment of viral infections in humans and animals. According to Vahidnia *et al.* [1], the viral infections are recognized as one of the leading causes of mortality across the globe. Although several antiviral therapeutic modalities are available, most of them lack specificity for particular viruses. Therefore, the development of novel antiviral treatments that are both cost-effective and specific has become the prime focus of current medical research. Viruses such as Influenza, in its various forms including pandemic, zoonotic and seasonal, continue to pose a significant threat to human health and have widespread economic implications. According to reports, from February 2003 to November 2009, there were 444 cases of avian influenza A (H5N1) virus infection in humans, resulting in 262 fatalities across 15 countries. The overall case fatality rate was alarmingly high, standing at 61.5% [2]. A systematic review conducted in 2018 analyzed 100 case studies published between 1995 and 2018 and estimated that globally, around 109.5 million influenza virus episodes (uncertainty range: 63.1-190.6), 10.1 million influenza virus associated ALRI cases (6.8-15.1), 870,000 influenza virus associated ALRI hospital admissions (543,000-1,415,000), 15,300 in-hospital deaths (5800-43,800) and up to 34,800 (13,200-97,200) overall influenza virus-associated ALRI deaths occurred among children under the age of 5 in 2018 [3].

The adamantanes (amantadine and rimantadine) were the first antiviral drugs to be approved for combating avian influenza A viruses as tricyclic amines. However, their overuse and unauthorized applications in domesticated poultry are believed to be the primary cause behind the emergence of drug-resistant strains. Drugs such as amantadine (ATD) often used as an antiviral medication is primarily used to treat and prevent influenza A virus infections. It works by preventing the virus from entering human cells and reproducing. However, when taken in high

doses, ATD can cause toxicity in humans, which can result in symptoms like confusion, hallucinations and seizures. Antivirals that are commonly administered besides adamantanes include nucleoside agents, such as acyclovir (ACV) and ribavirin (Rbv). Acyclovir is primarily used to treat herpes and respiratory tract viral infections. It is sometimes used alongside adamantanes to alleviate influenza symptoms [4]. However, inappropriate use of acyclovir has led to the emergence of resistant strains in both human and veterinary medicine.

Amantadine (ATD), chemically known as adamantan-1-amine, $C_{10}H_{17}N$, with mass 151.14 g/mol and molecular weight of 151.25 g/mol. It is a weak base and can be protonated to form a positively charged ion. ATD is generally used to treat Parkinson's disease and influenza A virus infections. It is believed to work by blocking the activity of the M2 protein ion channel in the viral membrane, thus preventing the virus from replicating. Amantadine is also an NMDA receptor antagonist, which means it can reduce the symptoms of Parkinson's disease by decreasing the amount of glutamate in the brain. The drug has a half-life of 10-15 h and is metabolized in the liver, gastric fluid and urine [5]. On the other hand, antiviral acyclovir (ACV), chemically known as amino-1,9-dihydro-9-((2-hydroxyethoxy)-methyl)-3*H*-purin-6-one, $C_8H_{11}N_5O_3$, bearing molecular mass of 225.2 g/mol is a nucleoside analogue. Acyclovir works by inhibiting the viral DNA polymerase, which prevents the virus from replicating, moreover, this drug is well-tolerated with few side effects and can cause headaches, nausea and diarrhea in some individuals.

Thus far, several approaches have been used to reduce the potential risks linked to antivirals. The People's Republic of China's Ministry of Agriculture prohibited the use of amantadine, rimantadine, acyclovir, ribavirin and moroxydine in the production of food animals in 2005 with Announcement No. 560 [6]. Similar to this, the American Food and Drug Administration (FDA) issued an Order of Prohibition in 2006 prohibiting the use of adamantanes and other antivirals in chicken regardless of the label. However, there is still proof of the use of these antiviral medications in poultry farming, especially in chicken farming, probably because of influenza viruses going through mutation. Despite the misuse and evident toxic effects associated with the antivirals ACV and ATD, there are currently no established forensic protocols capable of detecting these antivirals in forensic relevant matrices such as saliva, urine and gastric fluid.

To enhance the volatility of compounds for gas chromatography or GC-MS/MS and to improve instrument sensitivity when using liquid chromatography (LC) or capillary electrophoresis (CE), derivatization is often necessary for certain drugs due to their specific structure and physico-chemical properties. The combination of triple quadrupole mass spectrometry with high-performance liquid chromatography is the most common method employed for residue analysis [7]. Continuing advancements in LC-MS/MS, remarkably ultra-high performance liquid chromatography (UHPLC), have led to the development of novel techniques for multi-class multi-residue analysis. These new approaches offer improved capabilities and efficiency in detecting and quantifying multiple residues simultaneously [8]. Due to its superior sensitivity and selectivity in comparison to other techniques, the LC-MS/MS approach enables the direct determination of compounds.

The solid phase extraction (SPE) approach is frequently employed in LC-MS/MS analysis to prepare samples, however it is a difficult and time-consuming process. An expeditious sample preparation technique known as QuEChERS was developed to tackle this issue. Reversed-dispersive solid-phase extraction (r-dSPE) is used and conventional sample preparation stages are combined or simplified. This strategy not only minimizes the sample size but also significantly reduces the number of reagents, materials, energy and pretreatment time needed. QuEChERS was originally created to analyses pesticides in fruits and vegetables, but it is now frequently used to analyses a wide range of substances and matrices, including foods with animal origin [9], multi-residue alkaloids analysis [10], antidepressant drugs quantification [11,12] and antiallergic drugs activity screening [13]. However, there is currently no available method using QuEChERS and LC-MS/MS for analyzing ATD and ACV in urine, saliva and gastric fluid samples.

This work focuses and driven towards the quantification of ACV and ATD antiviral drugs using QuEChERS extraction and detection using LC-MS/MS form simulated saliva, gastric fluid and urine samples. The use of simulated matrices has been shown to improve accuracy and reduce false positives. The method is cost-effective, easy to use and can be applied to a wide range of biological samples. This study contributes to the development of more sensitive and specific analytical methods that can be used to improve food safety and protect public health.

EXPERIMENTAL

The standard forms of the antivirals *viz.* acyclovir (ACV) and amantadine (ATD) were provided by NIAM Labs Pvt. Ltd., Noida, India. Analytical grade chemicals and reagents such as methanol, water, formic acid and ammonium formate solutions for HPLC were obtained from Sigma-Aldrich, based in St. Louis, MO, USA. The EN D-SPE salt pouch (Agilent: 5982-0650, Agilent Technologies, Inc., USA) along with sodium citrate tribasic dihydrate (1 g) and sodium citrate dibasic sesquihydrate (0.5 g) were used in the QuEChERS method. Furthermore, anhydrous magnesium sulfate, sodium chloride, sodium acetate and primary secondary amine (PSA) were also procured from Sigma-Aldrich, USA.

Internal standards and working solutions: The standard stock solutions of ACV and ATD was prepared using methanol as solvent and stored at -20 ºC for stability. The working solution was then prepared by diluting the stock solution with Milli-Q water and stored at -20 ºC. The internal standard (IS) was also prepared in a similar manner. Subsequently, a series of working solutions with concentrations ranging from 5 to 200 ng/mL were prepared by diluting the stock solution in distilled water.

Preparation of simulated matrices: The artificial urine sample was prepared based on the procedure described by Stolarz *et al.* [14] with a total of eight components. Urea was used as

the only organic component, resulting in a concentration of 17 g/L. Additionally, the sample included chlorine at a concentration of 9.60 g/L, sodium at 5.40 g/L, sulphate at 1.35 g/L, magnesium at 0.65 g/L, calcium at 0.20 g/L and potassium at 0.20 g/L. These components were combined with 1 L of distilled water, resulting in a pH of 6.0. To mimic saliva, the method outlined by Pietrzyñska & Voelkel [15] was followed. The entire contents were dissolved in distilled water to obtain a final volume of 1 L. For the artificial gastric juice, the US Pharmacopeia protocol was employed [16]. This involved using 0.03 M aqueous NaCl, 0.084 M aqueous HCl and 0.32% (w/v) pepsin.

QuEChERS extraction: To optimize the QuEChERS extraction process, a two-step approach was employed. In first step, synthetic samples of saliva, urine and gastric fluid spiked with ACV and ATD at concentrations ranging from 5 to 200 ng/mL were prepared, respectively. A diluent containing 10 mL methanol and 10 mL Milli-Q water was added to the samples. The mixture was homogenized for 10 min on a wrist action shaker, followed by the addition of an EN QuEChERS salt pouch. After vortexing for 1 min, the mixture was centrifuged at 6000 rpm for 10 min at 2-8 ºC and 6 mL of supernatant was collected in a 15 mL centrifuge tube. In second step, the collected supernatant underwent a cleanup process. It was mixed with 500 mg of $MgSO_4$ and 250 mg of primary and secondary amine (PSA) to simplify the compound. After vortexed for 2 min and centrifuged at 6000 rpm for 6 min at 2-8 ºC, the extract was transferred to a tube containing 150 mg of $MgSO_4$ to further enhance the cleanliness of the extract. A volume of 200 µL of the resulting clean extract was then transferred to a different vial and 10 µL from each sample was directly injected into the LC-MS/MS apparatus for analysis. The study utilized synthetic samples and followed standard procedures for QuEChERS extraction and LC- MS/MS analysis, ensuring consistency and reliability in the experimental process.

Analysis procedure: LC-MS/MS analysis was performed using an Agilent 6470B instrument (Agilent Technologies, USA) operating in positive-ion mode to detect ACV and ATD from the matrices individually. A Poroshell 120 analytical column with an EC-C18 bonded phase (2.7 µm, 3 mm × 150 mm) was maintained at 40 ºC and the flow rate was set at 0.4 mL/min. The mobile phase consisted of 50 mM ammonium formate pH 2.9 (solvent A) and 0.9% formic acid in acetonitrile (solvent B). The following gradient elution program was applied: 0 min-90% B, 4.5 min-40% B, 8.5 min-40% B, 20 min-90% B. The initial conditions were restored in 5 min, resulting in a total run time of 25 min. The flow rate of the mobile phase was maintained at 0.2 mL/min and the injection volume was 10 µL. The MS source was set at a temperature of 250 ºC, with a nitrogen gas flow rate of 13 L/min and a nebulizer pressure of 30 psi.

Computational toxicity prediction: The computational toxicity prediction for ACV and ATD were performed on validated toxicity predictors such as ADMETlab 2.0, STopTox 1.0 and Syntelly. The canonical SMILES format for both the antivirals were retrieved from PubChem database and fed into the webservers. The toxicity prediction included a selection of multiple toxicity endpoints available on these platforms.

RESULTS AND DISCUSSION

To ensure the optimal performance, it is crucial to carefully evaluate and optimize specific parameters of QuEChERS method, such as the choice of solvent for extraction, the sample-to-solvent ratio, pH levels, agitation methods and the type and amount of partition salts and cleaning sorbents utilized [17-19]. Such investigations play a vital role in enhancing the precision and reliability of the analysis, while also improving the sensitivity and specificity of method. Additionally, the versatility of the QuEChERS method allows for modifications of traditional sample preparation approaches, making it a flexible and valuable tool in analytical chemistry [11,12].

To optimize the extraction conditions, a designed experiment was carried out to evaluate the QuEChERS method in two stages. Methanol was chosen as the solvent of choice over acetonitrile due to its gentle protein precipitation, water miscibility properties and effective solubility of both the antivirals, ACV and ATD [11]. In the dispersive solid-phase extraction (d-SPE) step, the inclusion of sorbents such as PSA, along with the addition of $MgSO_4$, facilitated the elimination of co-extracted components and helped remove residual water or salts from the samples.

The optimization process of QuEChERS method involved the incorporation of all analytes and their significant impacts *via* a numerical optimization approach. This method relied on the desirability concept, which combines the effects of factors and responses to produce the best final conditions. The optimal conditions, which aimed at achieving a desirable level of 98.3%, included the use of methanol as solvent, vortex or homogenizer agitation and PSA and $MgSO_4$ (1:6) (w/w) as sorbent cleanup. Additionally, partitioning salts such as sodium acetate and $MgSO_4$ (1:4) (w/w) were used. The optimized QuEChERS extraction parameters obtained through the design of experiments were subsequently used in the validation of technique.

Optimization of LC-MS/MS parameters: The LC-MS/MS system was employed to establish the optimal chromatographic and spectrometric conditions for both ACV and ATD antiviral drugs by introducing clean standard solutions into the system. Initially, precursor ions were detected and subsequently, product ions were identified using various collision energy voltages. Among these transitions, one was chosen as the quantifier ion, while the peak with the second-highest intensity was designated as the qualifier ion. To ensure accuracy, multiple reactions monitoring (MRM) transitions and dwell periods were automatically adjusted based on these parameters.

The mobile phase composition, including buffer concentration and pH, plays a pivotal role in enhancing quantification specificity and MS conditions as it directly influences the peak shape of analytes during chromatography and their ionization efficiency in MS. Upon the addition of 5 mM ammonium formate, a decrease in peak width and an improvement in peak symmetry were observed. Furthermore, the signal response of ACV and ATD significantly improved with the addition of 0.1% formic acid. Additionally, methanol demonstrated superior elution performance compared to acetonitrile. As a result, a mobile phase consisting of methanol:5 mM ammonium formate:formic acid

(35:65:0.1, v/v/v) was chosen to maintain consistent sensitivity and effective retention of ACV and ATD, respectively.

The precursor/product ion mass transitions were measured at 226.10 → 152.00 and 152.0 → 135.1 for ACV and ATD, respectively in all three matrices and the optimal LC conditions for retention times in urine, saliva and gastric lavage were recorded for ACV and ATD, respectively (Table-1). The MS parameters were optimized using a 100 ng/mL tuning solution in positive and negative ionization modes. In positive ionization mode, a substantially higher sensitivity with minimal background noise was achieved compared to the negative mode for both drugs. Representative chromatograms of ACV and ATD from all three matrices, along with their respective retention times, are presented in Figs. 1-6.

Method validation

Selectivity: The chromatograms were carefully inspected to identify any potential interference peaks that could coincide with the retention time of the analytes. The examination revealed that there were no interferences caused by the presence of reagents or solvents at the retention time of either ACV or ATD. As a result, it is concluded that the analytical method employed in this study is highly selective for ACV and ATD, respectively.

Matrix and carryover effects: The presence of co-eluted matrix components can lead to a matrix effect, resulting in either ion suppression or enhancement. Such matrix effects can significantly impact the sensitivity and reproducibility of an analytical assay [20]. In this study, matrix effects were assessed for all analytes at two distinct concentration levels: lower quality control (LQC) of 5 ng/mL and higher quality control (HQC) of 200 ng/mL (Table-1). The study found that the matrix effects for all analytes were within a variation of less than 20% at both quality control levels, which meets the validation standards and is considered acceptable.

The method demonstrated selectivity for both ACV and ATD, as no interference was detected in simulated urine, saliva and gastric lavage samples during the interference test. Moreover, blank samples of drug-free biological matrices were

TABLE-1
LIMIT OF DETECTION (LOD), LIMIT OF QUANTIFICATION (LOQ), MATRIX EFFECT (ME) AT LQC AND HQC AND OTHER METHOD VALIDATION PARAMETERS

Drug	Matrices	Slope	Intercept	R^2	LOD (ng/mL)	LOQ (ng/mL)	ME-LQC (%CV)	ME-HQC (%CV)	Precursor (*m/z*)	Product (*m/z*)	RT
Acyclovir	Gastric fluid	992	4291	0.998	17.91	54.27	14.32	-4.02	226.10	152.00	5.265
	Saliva	1061	5051	0.997	19.80	60.01	14.33	13.88	226.10	152.00	5.251
	Urine	991	4268	0.998	17.98	54.49	14.34	-4.49	226.10	152.00	5.265
Amantadine	Gastric fluid	4533	21517	0.997	20.85	63.16	-6.63	-1.06	152.00	135.1	5.917
	Saliva	3772	14382	0.999	14.06	42.61	8.18	3.72	152.00	135.1	5.895
	Urine	4532	21453	0.997	20.88	63.28	-10.04	-2.13	152.00	135.1	5.917

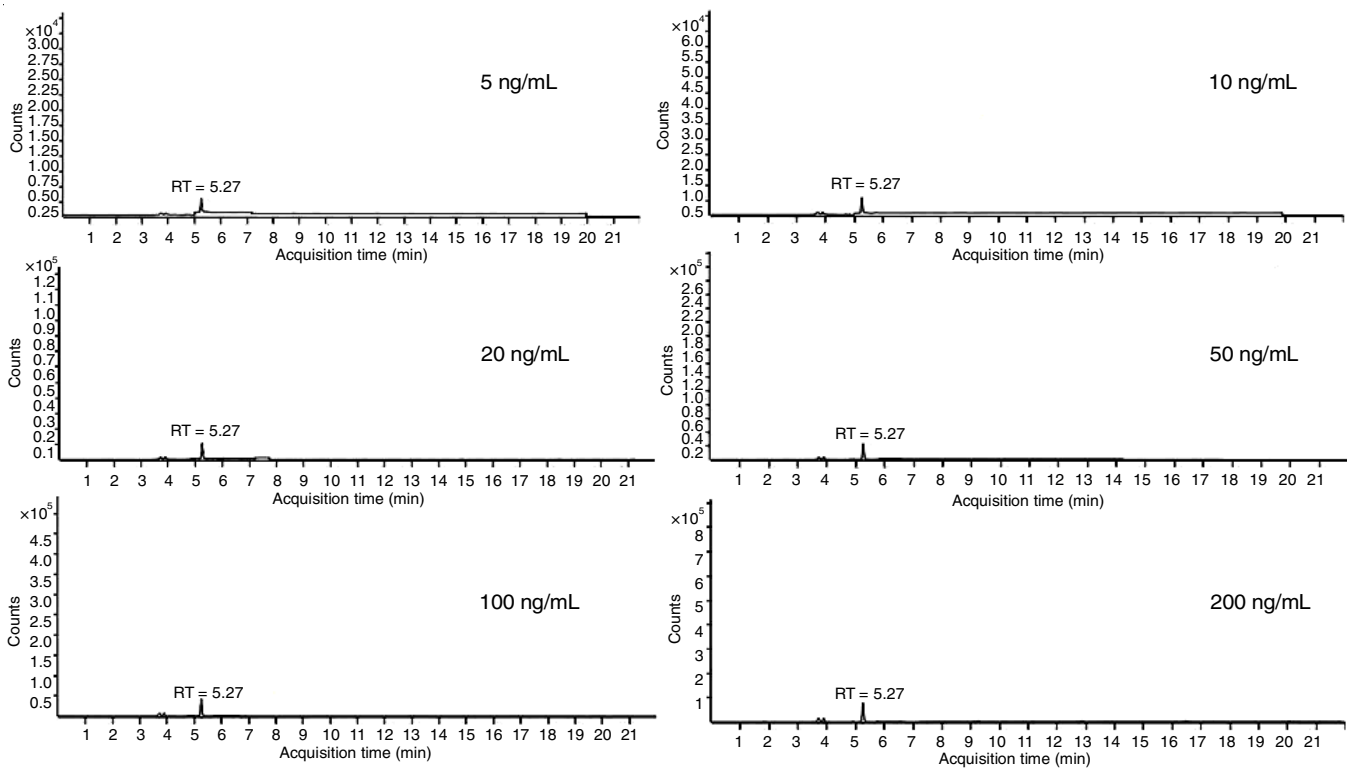

Fig. 1. LC-MS/MS chromatograms (acquisition time *vs*. counts) of acyclovir at 5 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL in urine

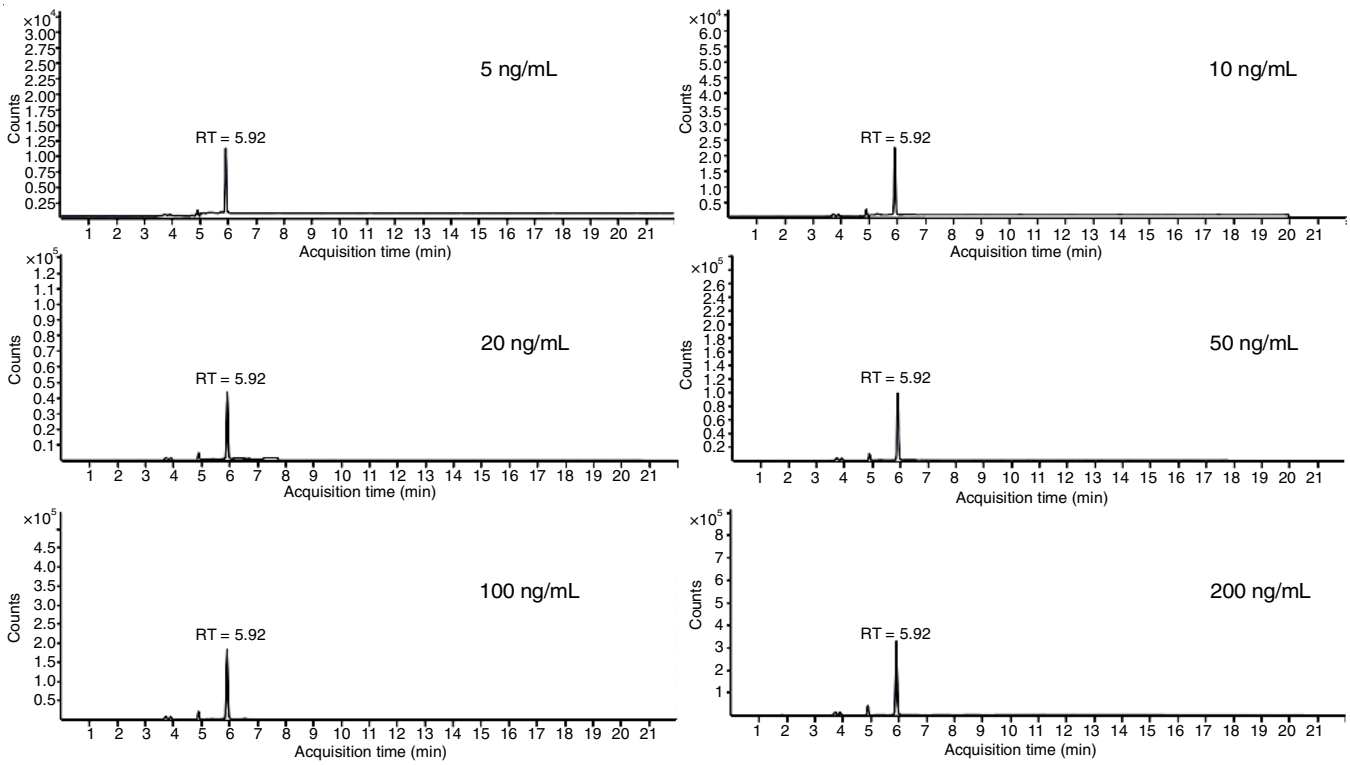

Fig. 2. LC-MS/MS chromatograms (acquisition time *vs*. counts) of amantadine at 5 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL in urine

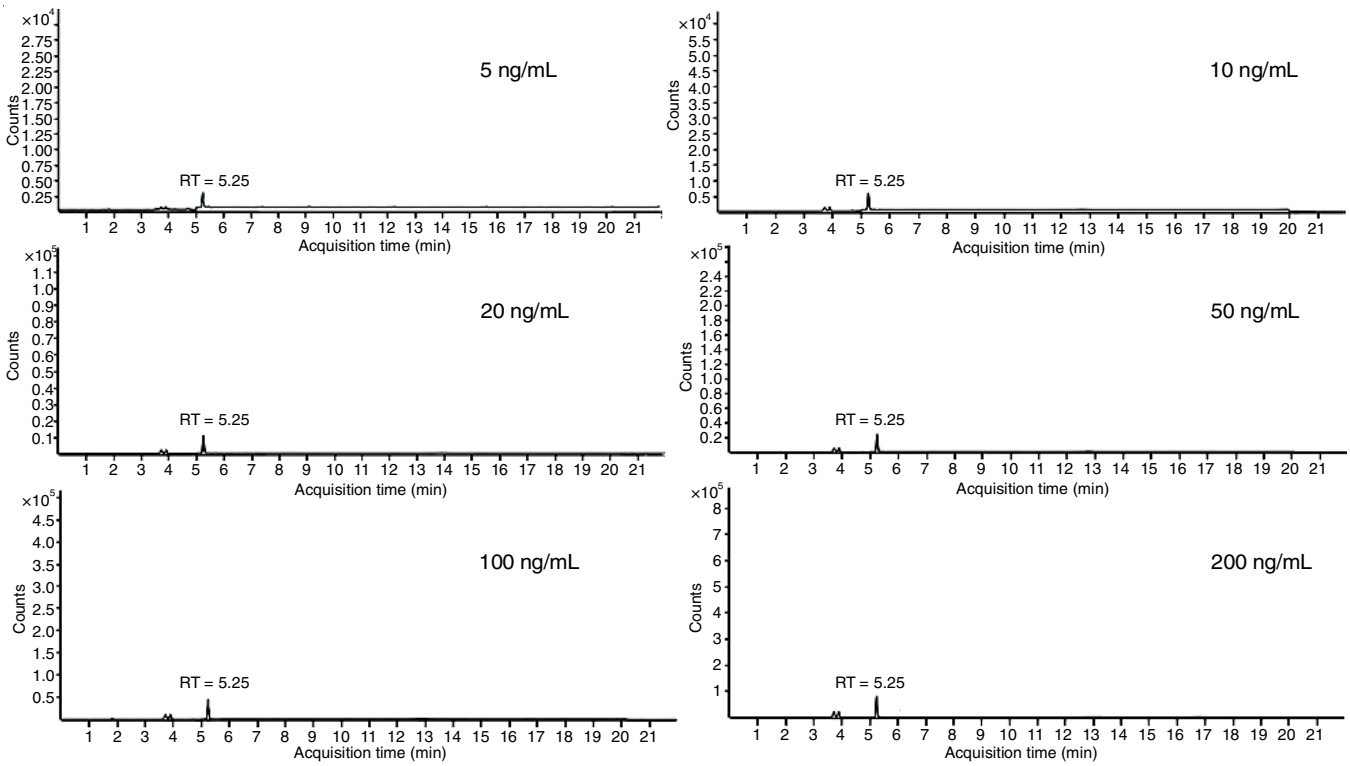

Fig. 3. LC-MS/MS chromatograms (acquisition time *vs*. counts) of acyclovir at 5 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL in saliva

analyzed using the adopted method to check for any carryover effects. Carryover was observed at the highest concentrations identified from earlier injections of the target drug. However, this carryover effect did not affect the accurate quantification of either ACV or ATD in subsequent samples. These findings confirm the reliability and robustness of the analytical method

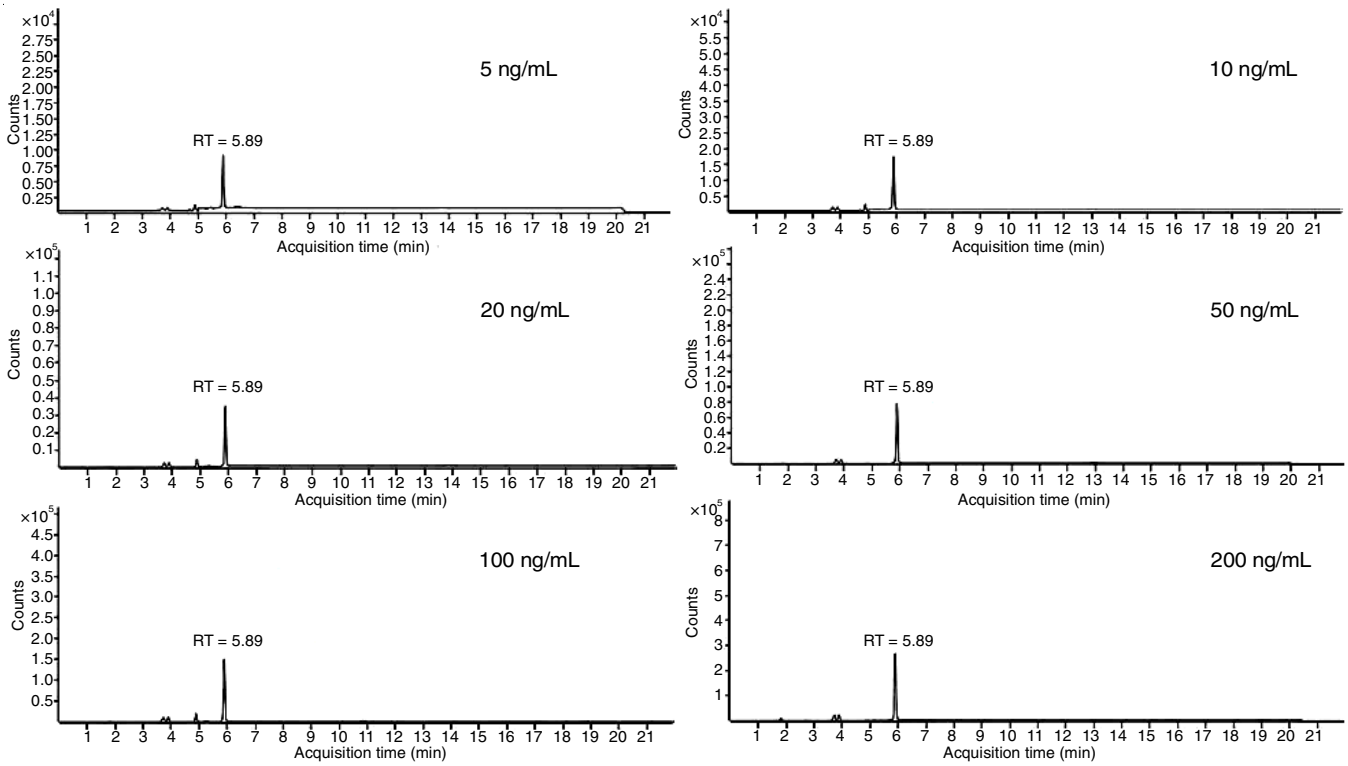

Fig. 4. LC-MS/MS chromatograms (acquisition time *vs*. counts) of amantadine at 5 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL in saliva

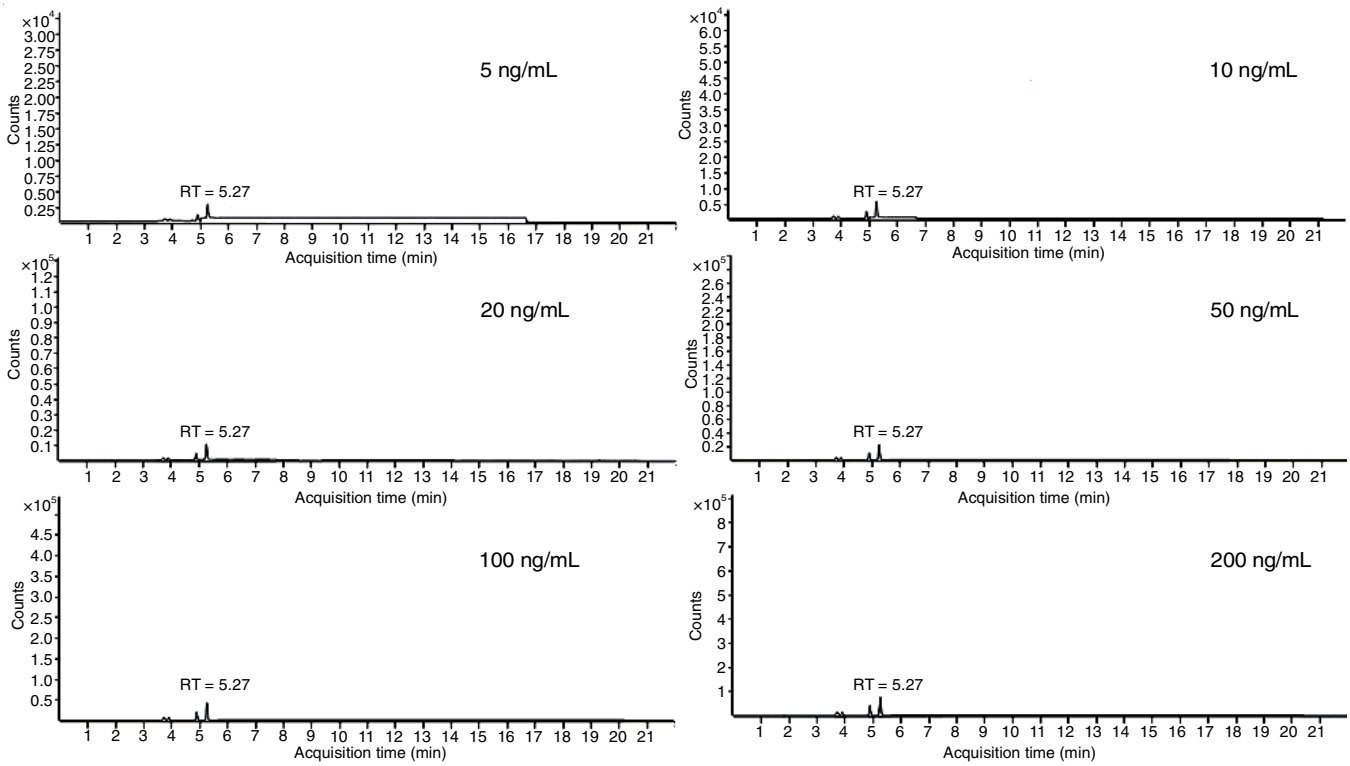

Fig. 5. LC-MS/MS chromatograms (acquisition time *vs*. counts) of acyclovir at 5 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL in gastric fluid

in accurately quantifying both ACV and ATD in various biological matrices. The results support the suitability of the method for use in forensic analysis and other related applications.

Linearity, limit of detection (LOD) and limit of quantification (LOQ): The calibration curve for the study was established by plotting the peak area against standard internal ratios

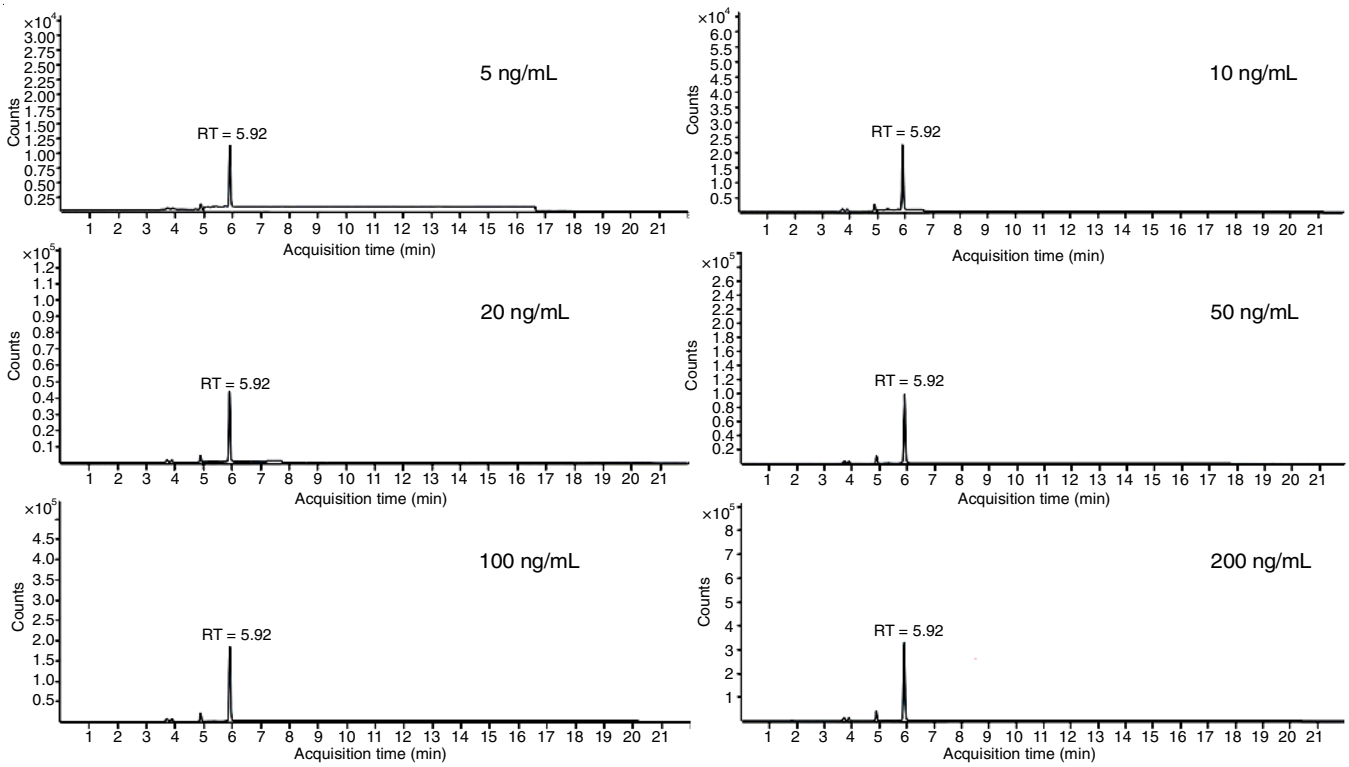

Fig. 6. LC-MS/MS chromatograms (acquisition time *vs*. counts) of amantadine at 5 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL in gastric fluid

of analytes at six different concentration levels. The limits of detection (LOD) and quantification (LOQ) were calculated and are listed in Table-1. The calibration range exhibited a linear curve with $1/x^2$ weighting and the linear regression coefficient (R^2) exceeded 0.9999, indicating a highly reliable model fit.

Accuracy and precision: The precision and accuracy of the method were assessed by evaluating the repeatability (intra-day) and intermediate precision (inter-day) of the results, expressed as %CV (coefficient of variation). The obtained %CV values for ACV and ATD in all three matrices (Table-2) were found to be within the acceptable criteria (not exceeding 20%). This indicates that the method demonstrated reliable and consistent performance in quantifying ACV and ATD in the tested samples.

Recovery: To evaluate the recovery, two sets of samples were used: one set involved spiking the blank matrix after extraction (AE), while the other set was spiked before the extraction process (BE). The percent recovery was determined using the formula described in Singh *et al.* [11]. The calculated percent recoveries of ACV and ATD from urine, saliva and gastric lavage all fell within the desired range of 70-120%, indicating that the recovery achieved meets the criteria for bioanalytical methods (Table-2). This demonstrates the accuracy and reliability of the method in quantifying ACV and ATD in the specified matrices.

Computational toxicity prediction: ACV and ATD were subjected to *in silico* prediction software to evaluate their toxicity endpoints. The results suggested that ACV has the potential to be mutagenic and carcinogenic and it may lead to drug-induced liver injury, hepatotoxicity, developmental toxicity, eye and skin irritation and corrosion, among other toxic effects (Table-3).

TABLE-2
RECOVERY, PRECISION AND ACCURACY OF ACYCLOVIR AND AMANTADINE IN GASTRIC FLUID, SALIVA AND URINE

	Gastric fluid			Saliva			Urine		
	Recovery (%)	Precision (%)	Accuracy (%)	Recovery (%)	Precision (%)	Accuracy (%)	Recovery (%)	Precision (%)	Accuracy (%)
Acyclovir	100.53	17.64	-10.51	80.52	4.47	-23.14	80.53	3.76	-22.61
	105.82	1.23	4.37	117.13	7.05	8.70	102.32	2.29	2.42
	111.16	4.84	11.72	127.46	8.06	16.69	107.16	3.03	9.71
	99.04	4.43	3.82	114.46	4.99	10.23	98.04	14.83	-3.55
	94.61	4.38	-0.35	112.85	4.64	7.40	93.64	4.53	-1.35
	86.98	4.74	-7.99	103.19	5.32	-2.77	86.55	6.19	-6.79
Amantadine	76.76	7.79	-16.51	88.93	3.66	-14.28	73.96	6.21	-20.88
	96.99	3.49	0.31	103.02	1.89	0.82	95.79	2.88	-0.93
	109.96	0.62	9.25	112.31	1.64	11.19	104.81	3.39	8.91
	104.49	0.56	4.67	107.18	1.72	6.09	104.43	2.18	6.97
	101.24	1.32	1.56	103.07	1.12	2.31	100.24	1.94	2.53
	92.67	1.66	-5.57	97.15	1.28	-3.96	91.67	1.85	-6.54

Conversely, ATD demonstrated acute toxic effects including inhalation, oral and dermal toxicities and it also exhibited the potential to act as a carcinogen. Moreover, ATD can induce hepatoxicity and respiratory toxicity within the host (Table-3). Apart from the toxicological assessment conducted in this study, numerous published studies and case reports have highlighted the evident toxic effects induced by these antivirals. These effects include mitochondrial toxicity, nephrotoxicity, DNA damage and intriguingly, neurotoxic effects [21-23] resembling those caused by antidepressants and other drugs of abuse. Such findings emphasize the critical need for a forensic detection protocol to identify and quantify these antivirals from biological matrices.

In this study, the LC-MS/MS method was optimized to detect the presence of ACV and ATD individually in urine, saliva and gastric lavage matrices. Several workers [24-28] have reported various protocols for determining both ACV and ATD in human plasma, utilizing different techniques, solvents, salt combinations and SPE sorbents for the extraction process. In present study, a modified QuEChERS method was adopted using methanol as solvent instead of acetonitrile, to extract the antivirals from the selected matrices. The modified approach demonstrates that methanol is the most suitable solvent for QuEChERS applications and is effective for a wide range of analytes similar to the antivirals studied. Furthermore, the optimized method shows good recovery of both ACV and ATD at ng/mL levels from urine, saliva and gastric lavage matrices using LC-MS/MS.

Conclusion

The design of antiviral drugs, while targeting host cells, can inadvertently cause adverse effects ranging from mild to severe, making it a critical aspect of public health assessment. However, there is a lack of forensic protocols to detect individual toxicity, drug-drug interactions, overdose and drug abuse related to antivirals. This study aimed to develop a modified QuEChERS and LC-MS/MS technique to quantify antivirals acyclovir (ACV) and amantadine (ATD) accurately from three forensically important matrices *viz*. urine, saliva and gastric lavage. The QuEChERS pre-treatment method offered several advantages, including faster and simpler extraction compared to previously reported methods that utilized SPE columns. The optimized QuEChERS extraction method ensured low sample and solvent consumption, employing methanol as solvent, sodium acetate and magnesium sulphate salts for the salting-out effect and primary secondary amine (PSA) with $MgSO_4$ for sorbent clean-up. The method was thoroughly validated to meet stringent analytical requirements, including specificity, accuracy, linearity, intermediate precision and repeatability. The recovery rates ranged from 71-120%, with standard deviation values less than 20%, further affirming the reliability of method. Application of the approach to simulated biological samples demonstrated its ease, efficiency and accuracy in detecting low concentrations of ACV and ATD. This highlights its potential use in therapeutic monitoring and forensic analysis, addressing the pressing need for a comprehensive and reliable protocol for identifying these antivirals in forensically relevant samples. Therefore, this developed protocol addresses the issues related to antiviral drug abuse, overdose and fatalities more effectively, aiding in forensic investigations and public health assessments.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. F. Vahidnia, S.L. Stramer, D. Kessler, B. Shaz, G. Leparc, D.E. Krysztof, S.A. Glynn and B. Custer, *Qual. Life Res.*, **26**, 349 (2017); https://doi.org/10.1007/s11136-016-1392-5
2. S. Briand, J. Tam and M. Bloch and E. Mumford, Proc. Vaccinol., 2, 159 (2010); https://doi.org/10.1016/j.provac.2010.07.009

TABLE-3
TOXICITY PREDICTORS OF ACYCLOVIR AND AMANTADINE

Classification	Target	Toxicity prediction (antivirals)	
		Acyclovir	Amantadine
Toxicity endpoints	Mutagenicity	Yes	No
	Carcinogenicity	Yes	Yes
	Immunotoxicity	No	No
	Cytotoxicity	Yes	No
	Developmental	Yes	No
Organ	Hepatotoxicity	Yes	Yes
	Respiratory toxicity	No	Yes
	Drug induced liver injury	Yes	No
In vivo parameters	Mouse oral LD_{50}	2126 mg/kg	373 mg/kg
	Mouse intravenous LD_{50}	442 mg/kg	37 mg/kg
	Rat oral LD_{50}	2636 mg/kg	631 mg/kg
	Rat intravenous LD_{50}	737 mg/kg	50 mg/kg
Acute inhalation toxicity		No	Yes
Acute oral toxicity		No	Yes
Acute dermal toxicity		No	Yes
Eye irritation and corrosion		Yes	No
Skin sensitization		No	Yes
Skin irritation and corrosion		No	Yes

3. X. Wang, Y. Li, K.L. O'Brien, S.A. Madhi, M.A. Widdowson, P. Byass, S.B. Omer, Q. Abbas, A. Ali, A. Amu, E. Azziz-Baumgartner, Q. Bassat, W. Abdullah Brooks, S.S. Chaves, A. Chung, C. Cohen, M. Echavarria, R.A. Fasce, A. Gentile, A. Gordon, M. Groome, T. Heikkinen, S. Hirve, J.H. Jara, M.A. Katz, N. Khuri-Bulos, A. Krishnan, O. de Leon, M.G. Lucero, J.P. McCracken, A. Mira-Iglesias, J.C. Moïsi, P.K. Munywoki, M. Ourohiré, F.P. Polack, M. Rahi, Z.A. Rasmussen, B.A. Rath, S.K. Saha, E.A. Simões, V. Sotomayor, S. Thamthitiwat, F.K. Treurnicht, M. Wamukoya, L.M. Yoshida, H.J. Zar, H. Campbell, H. Nair, H. Nair, H. Campbell, X. Wang, Y. Li, A. Chung, M. Rahi, Q. Abbas, A. Ali, Z.A. Bhutta, B. Saeed, S.B. Soofi, M.T. Yousafzai, A.K. Zaidi, A. Amu, E. Awini, E. Azziz-Baumgartner, H.C. Baggett, S.S. Chaves, N. Shang, S.J. Schrag, M.-A. Widdowson, S. Tempia, Q. Bassat, M. Lanaspa, S. Acácio, W.A. Brooks, A. Driscoll, M.D. Knoll, K.L. O'Brien, C. Prosperi, A.H. Baqui, L. Mullany, P. Byass, C. Cohen, A. von Gottberg, O. Hellferscee, F.K. Treurnicht, S. Walaza, D. Goswami, M. Rahman, N.E. Connor, S. El Arifeen, M. Echavarria, D.N. Marcone, N. Reyes, A. Gutierrez, I. Rodriguez, O. Lopez, D. Ortiz, N. Gonzalez, A. Gentile, M. del Valle Juarez, A. Gordon, C. Cutland, M. Groome, S.A. Madhi, M.C. Nunes, S. Nzenze, T. Heikkinen, S. Hirve, S. Juvekar, N. Halasa, J.H. Jara, C. Bernart, M.A. Katz, I. Gofer, Y.S. Avni, N. Khuri-Bulos, S. Faori, A. Shehabi, A. Krishnan, R. Kumar, R. Amarchand, C.L. Contreras, O. de Leon, M.R. Lopez, J.P. McCracken, H. Maldonado, A.P. Samayoa, A.B. Gomez, M.G. Lucero, L.T. Nillos, S.P. Lupisan, H. Nohynek, A. Mira-Iglesias, J. Puig-Barberà, J. Díez-Domingo, B.D. Gessner, B.-M. Njanpop-Lafourcade, J.C. Moïsi, P.K. Munywoki, H. Tall, M. Ngama, D.J. Nokes, S.B. Omer, D.R. Clark, M. Ourohiré, S. Ali, Z. Pascal, B.H. Cheik, M.T. Caballero, R. Libster, F.P. Polack, Z.A. Rasmussen, E.D. Thomas, J.M. Baker, B.A. Rath, P.E. Obermeier, M. Hassanuzzaman, M. Islam, M.S. Islam, S.K. Saha, P. Panigrahi, A. Bose, R. Isaac, D. Murdoch, P. Nanda, S.A. Qazi, D. Hessong, E.A.F. Simões, V. Sotomayor, S. Thamthitiwat, M. Chittaganpitch, H. Dawood, C. Kyobutungi, M. Wamukoya, A.K. Ziraba, L.-M. Yoshida, D.-A. Dand, K. Yoshihara, M.-N. Le, M.P. Nicol, H.J. Zar, S. Broor, M. Chadha, L. Madrid, L. Gresh, A. Balmaseda, G. Kuan, N. Wairagkar, M.D. Tapia, S.L. Knobler, A. Barahona, E. Ferguson and B. Schweiger, *Lancet Glob. Health*, **8**, e497 (2020); https://doi.org/10.1016/S2214-109X(19)30545-5
4. K. Kuroda, C. Li, K. Dhangar and M. Kumar *Sci. Total Environ*., **776**, 145740 (2021); https://doi.org/10.1016/J.SCITOTENV.2021.145740
5. V.G. Vernier, J.B. Harmon, J.M. Stump, T.E. Lynes, J.P. Marvel and D.H. Smith *Toxicol. Appl. Pharmacol.*, **15**, 642 (1969); https://doi.org/10.1016/0041-008X(69)90066-0
6. B.J.A. Berendsen, R.S. Wegh, M.L. Essers, A.A.M. Stolker and S. Weigel, *Anal. Bioanal. Chem.*, **402**, 1611 (2012); https://doi.org/10.1007/s00216-011-5581-3
7. J.P. Yuan, Y.M. Sun, J.H. Liu, Y.X. Yao and Y. Chen, *J. Sep. Sci.*, **39**, 2846 (2016); https://doi.org/10.1002/jssc.201600005
8. Z.C. Liu, F. Yang, M. Yao, Y.H. Lin and Z.J. Su, *J. Sep. Sci.*, **38**, 1784 (2015); https://doi.org/10.1002/jssc.201401461
9. B.J. Kim, S.-H. Yang and H. Choi, *Foods*, **11**, 3634 (2022); https://doi.org/10.3390/foods11223634
10. A. Steinborn, L. Alder, M. Spitzke, D. Dörk and M. Anastassiades, *J. Agric. Food Chem.*, **65**, 1296 (2017); https://doi.org/10.1021/acs.jafc.6b05407
11. C. Singh, J. Sharma, B.P. Nayak, P. Pandya and H. Khajuria, *Biointerf. Res. Appl. Chem.*, **13**, 448 (2023).
12. C. Singh, J. Sharma, B.P. Nayak, P. Pandya, G.K. Singh and H. Khajuria, *Mater. Today Proc.*, (2023); https://doi.org/10.1016/j.matpr.2023.02.134
13. W. Arpornchayanon, S. Klinprung, S. Chansakaow, N. Hanprasertpong, S. Chaiyasate, M. Tokuda and H. Tamura, *Asian Pac. J. Allergy Immunol.*, **40**, 393 (2022); 10.12932/AP-300319-052912932/AP-300319-0529
14. A. Stolarz, A. Alonso, W. De Bolle, H. Kühn, S. Richter, C. Quetel, E. Ponzevera, A. Verbruggen, R. Wellum, Ec.Europa.Eu (2015).
15. M. Pietrzyñska and A. Voelkel, *Microchem. J.*, **134**, 197 (2017); https://doi.org/10.1016/j.microc.2017.06.004
16. U.S. Pharmacopeia and National Formulary, United States Pharmacopeia Convention Inc., Rockville, MD (2003).
17. R. Mahdavi and Z. Talebpour, *Trends Analyt. Chem.*, **160**, 116964 (2023); https://doi.org/10.1016/j.trac.2023.116964
18. S.J. Lehotay, *Methods Mol. Biol.*, **747**, 65 (2011); https://doi.org/10.1007/978-1-61779-136-9_4
19. R. Perestrelo, P. Silva, P. Porto-Figueira, J.A.M. Pereira, C. Silva, S. Medina and J.S. Câmara, *Anal. Chim. Acta*, **1070**, 1 (2019); https://doi.org/10.1016/j.aca.2019.02.036
20. D. Mariño and N. Patiño, *J. Anal. Toxicol.*, **46**, 37 (2022); https://doi.org/10.1093/jat/bkaa182
21. J.M. Barbara and A. Pace, *Pract. Neurol.*, **24**, e1 (2024); https://doi.org/10.1136/pn-2023-003723
22. D. Gruca, K. Antoniak, M. Wais and M. Zajac, *J. Educ. Health Sport*, **13**, 85 (2023); https://doi.org/10.12775/JEHS.2023.13.03.012
23. D. Brandariz-Nuñez, M. Correas-Sanahuja, S. Maya-Gallego and I. Martín Herranz, *J. Clin. Pharm. Ther.*, **46**, 918 (2021); https://doi.org/10.1111/jcpt.13464
24. J.J. Sasanya, A.M.M. Abd-Alla, A.G. Parker and A. Cannavan, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **878**, 2384 (2010); https://doi.org/10.1016/j.jchromb.2010.07.008
25. A. Bhadoriya, S. Rathnam, B. Dasandi, D. Parmar, M. Sanyal and P.S. Shrivastav, *J. Pharm. Anal.*, **8**, 202 (2018); https://doi.org/10.1016/j.jpha.2017.10.003
26. K. Wang, M. Chen, H. Weng, Y. Gao, H. Zhao and Z. Lin, *J. Appl. Bioanal.*, **4**, 51 (2018); https://doi.org/10.17145/jab.18.008
27. M.A. Farajzadeh, N. Nouri and A.A. Alizadeh Nabil, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **940**, 142 (2013); https://doi.org/10.1016/j.jchromb.2013.09.035
28. M. Saraji, T. Khayamian, S. Mirmahdieh and A.A.H. Bidgoli, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **879**, 3065 (2011); https://doi.org/10.1016/j.jchromb.2011.09.017