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RESEARCH ARTICLE

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Liquid chromatography–tandem MS/MS method for simultaneous quantification of paracetamol, chlorzoxazone and aceclofenac in human plasma: An application to a clinical pharmacokinetic study

Dalia Mohamed[1,2] | Maha A. Hegazy[3] | Mona S. Elshahed[1] | Safaa S. Toubar[1] | Marwa I. Helmy[1]

[1] Analytical Chemistry Department, Faculty of Pharmacy, Helwan University, Cairo, Egypt

[2] Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, October University for Modern Sciences and Arts, 6 October City, Egypt

[3] Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt

Correspondence
Mona S. Elshahed, Analytical Chemistry Department, Faculty of Pharmacy, Helwan University, Cairo, Egypt.
Email: monaelshahed@gmail.com

Funding information
Helwan University

Abstract

A facile, fast and specific method based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the simultaneous quantitation of paracetamol, chlorzoxazone and aceclofenac in human plasma was developed and validated. Sample preparation was achieved by liquid–liquid extraction. The analysis was performed on a reversed-phase C_{18} HPLC column (5 μm, 4.6 × 50 mm) using acetonitrile–10 mM ammonium formate pH 3.0 (65:35, *v*/*v*) as the mobile phase where atrovastatin was used as an internal standard. A very small injection volume (3 μL) was applied and the run time was 2.0 min. The detection was carried out by electrospray positive and negative ionization mass spectrometry in the multiple-reaction monitoring mode. The developed method was capable of determining the analytes over the concentration ranges of 0.03–30.0, 0.015–15.00 and 0.15–15.00 μg/mL for paracetamol, chlorzoxazone and aceclofenac, respectively. Intraday and interday precisions (as coefficient of variation) were found to be ≤12.3% with an accuracy (as relative error) of ±5.0%. The method was successfully applied to a pharmacokinetic study of the three analytes after being orally administered to six healthy volunteers.

KEYWORDS

aceclofenac, chlorzoxazone, HPLC–MS/MS, human plasma, paracetamol, pharmacokinetic study

1 | INTRODUCTION

Multicomponent dosage forms are widely used owing to their great tolerability, multiple actions, synergistic effects and patient acceptance. The combination of paracetamol (PAR), chlorzoxazone (CXZ) and aceclofenac (ACL) is emerging as a widely prescribed combination in a single dosage form which is used as muscle relaxant medication. PAR [*N*-(4-hydroxyphenyl) acetamide] has analgesic and antipyretic properties and weak anti-inflammatory activity. It is often the analgesic or antipyretic of choice, especially in the elderly, children and in those with a history of peptic ulcer. It is a major component in numerous cold and flu preparations also in combination with nonsteroidal anti-inflammatory drugs and opioid analgesics (Sweetman, 2011). Surveying the literature revealed that PAR was determined in biological fluids either individually or in combination with other drugs by various analytical methods (Abro, Memon, Bhanger, Perveen, & Kandhro, 2012; Celma, Allue, Prunonosa, Peraire, & Obach, 2000; Farid & Abdelaleem, 2016; Gicquel, Aubert, Lepage, Fromenty, & Morel, 2013; Hairin et al., 2013; Hewavitharana, Lee, Dawson, Markovich, & Shaw, 2008; Li et al., 2010; Liao et al., 2008; Locatelli et al., 2015; Lou, Yuan, Ruan,

Abbreviations: ACL, aceclofenac; ATR, atrovastatin; CXZ, chlorzoxazon; ESI, electrospray positive and negative ionization; MRM, multiple-reaction monitoring; PAR, paracetamol.

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& Jiang, 2010; Ophelia, Sherry, & Moses, 2000; Trettin et al., 2011; Zhu, Ding, Guo, Yang, & Wen, 2007).

CXZ [5-chloro-2(3H)-benzoxazolone] is a skeletal muscle relaxant. It acts by inhibiting multisynaptic reflexes involved in producing and maintaining skeletal muscle spasm. It acts on the spinal cord by depressing reflexes. It also reduces the release of inflammatory leukotrienes and is used to relieve the pain and stiffness caused by muscle strains and sprains (Sweetman, 2011). Literature survey has revealed several methods for the determination of CXZ in biological fluids (Abbar & Nandibewoor, 2012; Eap, Schnyder, & Savary, 1998; Rajnarayana, Mada, Vidyasagar, Kishore, & Krishna, 2002; Simonsen, Steentoft, Buck, Hansen, & Linnet, 2010; Walash, Belal, Tolba, & Halawa, 2015; Wang et al., 2010; Zhao et al., 2014).

ACL {2-[(2, 6-dichloro phenylamino) phenyl] acetoxy-acetic acid} is an effective nonsteroidal anti-inflammatory drug, which possesses remarkable anti-inflammatory, analgesic and antipyretic properties. ACL appears to be particularly well-tolerated among the nonsteroidal anti-inflammatory drugs with a lower incidence of gastrointestinal adverse effects (Sweetman, 2011). A literature survey revealed some methods for the determination of ACL in pharmaceutical dosage forms and biological fluids (El-Bagary, Azzazy, ElKady, & Farouk, 2014; Hinz et al., 2003; Kang & Kim, 2008; Kim, Ahn, Noh, Kang, & Gwak, 2012).

A summary of the chromatographic conditions and the quantitation ranges of the cited reported HPLC–MS/MS methods for the determination of the studied drugs in human biological samples is demonstrated in Table 1. PAR and CXZ are official in the United States Pharmacopeia 36 (2013) while ACL is official in the European Pharmacopoeia (2014). The structures of the three drugs are presented in Figure 1. Survey of the literature revealed some reported analytical methods for the simultaneous determination of these drugs in their ternary mixture. The developed methods include spectrophotometric methods which were utilized for the analysis of the three drugs in their combined dosage form (Gare, Swarnlata, & Saraf, 2007; Toubar, Hegazy, Elshahed, & Helmy, 2016); these methods depend mainly on solving the overlapped drug spectra using mathematical calculations or instrument software. Although these methods are simple and of low cost and are capable of determining the drugs with high accuracy and precision, generally spectrophotometric methods lack the sensitivity for determining analytes in plasma. Several chromatographic methods were also developed for the simultaneous determination of the studied drugs, including high performance thin layer chromatography (Mahajan, Bari, Shirkhedkar, & Surana, 2008), High-performance liquid chromatography with ultraviolet detection (HPLC-UV; Joshi & Sharma, 2008; Hari et al., 2008; Karthikeyan, Vaidhyalingan, & Nema, 2009; Pawar, Naik, Sulebhavikar, Datar, & Mangaonkar, 2009; Rathinavel et al., 2010; Ravisankar, Devadasu, Devala, & Nageswara, 2013; Shaikh & Devkhile, 2008) and supercritical fluid chromatography (Desai, Patel, Sherikar, & Mehta, 2012). Although HPLC is a preferable method for the analysis of analytes in plasma, the employed methods have shown disadvantages which make them inappropriate for pharmacokinetic studies, including low sensitivity and high flow rate, thus consuming a lot of solvent, and long run time.

Analytical studies involving simultaneous multicomponent quantitation of these combinations in human plasma require the high specificity, sensitivity and efficiency of HPLC with fluorescence or mass spectrometric detection. When other factors are considered such as the widely different polarities of the drugs, the different classes of the drugs, different characters of the drugs and the desire for an analytical method that involves simple sample preparation and a rapid run time, method selection devolves onto liquid chromatography–tandem mass spectrometry (LC–MS/MS).

Thus, the objective of the present study is to develop and validate a reliable, economical and rapid LC–MS/MS method for the quantitative determination of PAR, CXZ and ACL simultaneously in human plasma and its application to a pharmacokinetic study in healthy volunteers after a single oral dose of their co-formulated tablets.

2 | EXPERIMENTAL

2.1 | Materials and reagents

Pure samples of PAR and CXZ were kindly supplied by EVA Pharma Pharmaceutical Company (Giza, Egypt). The purity of the samples was certified to be 100.6% and 100.4%, respectively, according to the official methods (The United States Pharmacopeia 36, 2013). ACL was kindly supplied by Amriya Pharmaceutical Company (Alexandria, Egypt). It had a certified purity of 100.8% according to the official method (European Pharmacopoeia, 2014). Atrovastatin internal standard (IS) was supplied by Sigma Aldrich (St Louis, MO, USA). It had a certified purity of 100.2% according to the official method (The United States Pharmacopeia 36, 2013).

Dolokind-MR® tablet, batch no. C1AGM016 (labeled to contain 325 mg PAR, 250 mg CXZ and 100 mg ACL) was manufactured by Mankind Pharma (Delhi, India) and was purchased from the market. All solvents and materials were of HPLC grade. Methanol (MeOH), methyl tertiary butyl ether (MTBE), ethyl acetate, dichloromethane (DCM) and diethyl ether (DE) were purchased form Fischer Scientific UK Ltd (Loughborough, UK). Acetonitrile (ACN), ammonium acetate and ammonium formate were purchased from Merck (Darmstadt, Germany). Deionized water (Purelab Flex, ELGA) was used. Human plasma was purchased from Vaccera (Giza, Egypt) and stored at −85°C.

2.2 | Instruments

Chromatographic separation was carried out using an Agilent 1200 HPLC system (Agilent Technologies, USA). Mass spectrometric detection and quantitative analysis were carried out using a triple quadrupole API 4000 (ABSciex, Canada), operated in positive and negative electrospray ionization modes. Hardware control and data acquisition and treatment were carried out using Analyst1.5.2 Software with Hotfixes to February 2011 (ABSciex, Canada).

2.3 | Liquid chromatographic and mass spectrometric conditions

Separations were carried out using a Waters Zorbax column SB C_{18} (5 μm, 4.6 × 50 mm) and a mobile phase of acetonitrile–10 mM ammonium formate (pH 3.0; 65:35, *v*/*v*). Isocratic elution with a flow rate of 0.6 mL/min and injection volumes of 3 μL were employed. A standard mixture of PAR, CXZ, ACL and IS (20.00 ng/mL each in methanol)

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TABLE 1 Comparison between the chromatographic conditions used for quantification of ACL, CXZ and PAR using HPLC–MS/MS reference methods and our proposed method

Analytes	Matrix	Mobile phase	Flow rate	Run time	Stationary phase	Quantitative range of our studied drug	Reference
ACL and its three metabolites	Human plasma	Acetonitrile–0.1% formic acid (80: 20, v/v)	0.2 mL/min	2.5 min	Zorbax C_{18} (100 × 2.1 mm, 3 μm)	0.1–20 μg/mL	Kang and Kim (2008)
CXZ with diclofenac, dapson, dextromethorphan, omeprazole and phenacetin	Human liver microsomal incubation mixtures	45% A–55% B (A, acetonitrile; B, 10 mM ammonium acetate with 0.1%formic acid)	Gradient (0–3.7 min at 0.2 mL/min; 3.7–9.0 min at 0.35 mL/min)	9.0 min	Waters Atlantis® C_{18} column (100 × 2.1 mm, 5 μm)	75.0–3000.0 ng/mL	Zhao et al. (2014)
CXZ with 11 acidic or neutral drugs	Whole blood	5 mM ammonium acetate (pH 8.5)–acetonitrile (2:3, v/v)	0.1 mL/min	9.0 min	Varian Pursuit C_{18} column (100 × 3 mm, 3 μm)	1–100 mg/kg	Simonsen et al. (2010)
PAR and its primary metabolites	Human plasma	Aqueous 1% formic acid–methanol (80:20, v/v)	0.2 mL/min	10.0 min	Hypersil Gold C_{18} column (100 × 2.1 mm, 3 mm)	0.25–20 mg/L	Gicquel et al. (2013)
PAR, pseudoephedrine and loratadine	Spiked plasma	Gradient elution with solvent A (methanol) and B (0.1% formic acid)	1.0 mL/min	12.0 min	Chromolith® Performance RP_{18e} column (100 × 4.6 mm)	0.039–10 μg/mL	Abro et al. (2012)
PAR, pseudoephedrine, dextrophan and chlorpheniramine	Human plasma	Gradient elution with 0.3% (v/v) acetic acid and methanol	0.3 mL/min	6.0 min	YMC-ODSAQ C_{18} column (100 × 2.0 mm, 3 μm)	10–5000 ng/mL for PAR	Lou et al. (2010)
PAR, caffeine, pseudoephedrine, chlorpheniramine and cloperastine	Human plasma	Formic acid–10 mM ammonium acetate–methanol (1:40:60, v/v/v)	1 mL/min	2.6 min	Venusil Mp-C_{18} column (50 × 4.6 mm, 5 μm)	5.0–2000 ng/mL	Li et al. (2010)
PAR, pseudoephedrine and chlorpheniramine	Human plasma	Methanol–acetonitrile–1% formic acid (60:20: 20, v/v/v)	0.3 mL/min	2.5 min	Aquasil-C_{18} column (150 × 2.1 mm, 5 μm)	20.0–10,000.0 ng/mL	Liao et al. (2008)
PAR and tramadol	Human plasma	10 mM ammonium acetate buffer containing 0.5% formic acid–methanol (40:60, v/v)	1 mL/min	5 min	LiChrospher CN column (250 × 4.6 mm, 5 μm)	0.03–16 μg/mL	Zhu et al. (2007)
PAR and chlorpheniramine	Human plasma	Water–acetonitrile (80: 20), 0.5% formic acid and 1 mM pentafluoropropionic anhydride	1 mL/min	4.5 min	Kromasil C column (50 × 4.6 mm, 5 μm)	0.5–25 μg/mL	Celma et al. (2000)
PAR, CXZ and ACL	Human plasma	Acetonitrile–10 mM ammonium formate pH 3.0 (65:35, v/v)	0.6 mL/min	2.0 min	Waters Zorbax column SB C_{18} (50 × 4.6 mm, 5 μm)	0.03–30.0 μg/mL, 0.015–15.00 μg/mL and 0.15–15.00 μg/mL for PAR, CXZ and ACL	Our proposed method

ACL, Aceclofenac; CXZ, chlorzoxazon; PAR, paracetamol.

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PAR CXZ ACL

FIGURE 1 Chemical structure of paracetamol (PAR), chloroxazone (CXZ) and aceclofenac (ACL)

was directly infused into the mass spectrometer, and the operating conditions were optimized.

ESI was operated in the positive-ion mode for PAR and ATR and negative-ion mode for CXZ and ACL. The quadrupole mass spectrometer operated in the MRM mode was used for monitoring the transition of molecular ions to the product ions for PAR *m/z* 152.3 → 110.0, CXZ *m/z* 167.9 → 131.9, ACL *m/z* 353.2 → 75.1 and IS *m/z* 559.4 → 440.3. The nebulizer gas was air (zero grade), whereas nitrogen was used as the auxiliary, curtain and collision gas. The source/gas-dependent parameters for PAR, CXZ, ACL and IS determination were as follows: curtain gas, 10 psi; collision gas, 10 psi; turbo ion spray temperature, 600°C; ion spray voltage, 5500 V for positive mode and − 4500 V for the negative mode, ion source gas one, 45 psi; and ion source gas two, 40 psi.

2.4 | Procedures

2.4.1 | Preparation of stock and working standard solutions

Primary stock standard solutions containing 300.0 μg/mL of PAR, 150.0 μg/mL of CXZ, 150.0 μg/mL of ACL and 20.0 μg/mL of ATR were prepared separately in methanol. Appropriate dilutions were made in methanol from the corresponding primary stock standard solutions to obtain working stock solutions (1) of 30.0 μg/mL for PAR, 15.0 μg/mL for both CXZ and ACL, and 2.0 μg/mL for ATR. Further dilutions in methanol from the corresponding working stock solutions (1) produced working stock solutions (2) of 3.0 μg/mL for PAR and 1.50 μg/mL for CXZ.

All standard solutions were stored at −20°C and were stable for about 1 month. Ten different working standard solutions were prepared by appropriate dilutions of accurately taken volumes from the above prepared primary stock and working stock solutions into 10-mL volumetric flasks with methanol.

2.4.2 | Preparation of calibration standards and quality control samples

Aliquots of 450 μL of blank human plasma were spiked with 50 μL of increasing concentrations of PAR, CXZ and ACL from their working standard solutions. The final concentrations in the plasma samples were 0.03, 0.06, 0.30, 0.60, 0.90, 3.0, 6.0, 9.0, 18.0 and 30.0 μg/mL for PAR, 0.015, 0.03, 0.15, 0.30, 0.45, 1.50, 3.0, 4.50, 9.0 and 15.0 μg/mL for CXZ and 0.15, 0.30, 0.90, 1.50, 3.0, 4.50, 9.0 and 15.0 μg /mL for ACL. The plasma calibration curve consisted of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard) and non zero samples covering the expected range, including the lower limit of quantification (LLOQ).

Similarly, quality control standards (QCs) used in the validation were prepared for the practically determined LLOQ with final concentrations of 0.03, 0.015 and 0.15 μg/mL, low quality control (LQC) with final concentrations of 0.09, 0.045 and 0.45 μg/mL, medium quality control (MQC) with final concentrations of 15.00, 7.50 and 7.50 μg/mL and high quality control (HQC) with final concentrations of 24.00, 12.00 and 12.00 μg/mL for each of PAR, CXZ and ACL, respectively.

Aliquots of 0.5 mL plasma were spiked with 50 μL of IS stock solution, and the mixture was vortex mixed for 1 min before and after addition of 50 μL of 25% acetic acid. Liquid–liquid extraction was then carried out by adding 3.0 mL (*tert*-butyl methyl ether) and samples were vortexed for 3 min then centrifuged at 3000 rpm at 2°C for another 2 min. The upper clear layer was carefully separated and evaporated under vacuum at 45°C, then reconstituted with 2 mL (methanol–water 7:3), and 3 μL was injected into the LC–MS/MS system. The peaks were interpreted based on the reported peak areas. Concentrations of PAR, CXZ and ACL in unknown samples were calculated by referring to the prepared calibration curves.

2.5 | Method validation

2.5.1 | Specificity

The specificity of the method was assessed using six different batches of blank human plasma randomly selected from different sources. Blank plasma samples were prepared and analyzed as described, and the peak areas were compared with those noted with a set of blank plasma samples containing the analytes at the LLOQ level.

2.5.2 | Linearity and range

The calibration spiked plasma samples were prepared as described in Section 2.4.2. The plasma calibration curves were constructed by plotting peak area ratios of each analyte to that of IS against the corresponding concentrations. For acceptable results, the correlation coefficient (r) of the calibration curve should be ≥0.99 and the back-calculated concentrations at each point have to be within ±15% from the nominal value, except at LLOQ where deviation is acceptable up to ±20%.

2.5.3 | Precision and accuracy

Inter- and intra-assay precision and accuracy were determined by examining six replicates at the LLOQ in addition to three different QC levels as described previously on the same and different days. Intra- and interday precision were assessed by the relative standard deviation (CV) at each concentration level. However, accuracy was evaluated by calculating the relative error (RE). For acceptable precision and accuracy, variation should be within 15%, except for the LLOQ, where the value of the variation was permitted to be up to 20%.

2.5.4 | Recovery

The recoveries for PAR, CXZ and ACL were calculated by comparing the responses of the analytes extracted from replicate QC samples (low, medium and high) with the response of analytes from post-extracted plasma standard sample (i.e. spiking is performed after plasma extraction) at equivalent concentrations.

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2.5.5 | Matrix effects

To evaluate the effect of plasma constituents on the ionization of analytes and IS, comparison of the responses of the post extracted plasma standard QC samples ($n = 4$) with the response of analytes from neat samples at equivalent concentrations was carried out.

2.5.6 | Stability experiments

- *Short-term stability* was studied by examining triplicate of LQC, MQC and HQC samples, where these samples were thawed at room temperature and kept for a period of 6 h before analysis.
- *Freeze–thaw stability* was investigated by examining the stability of QC samples over three freeze–thaw cycles. Triplicate frozen LQC, MQC and HQC samples were allowed to thaw at room temperature for more than 2 h and then frozen at −86°C overnight for each cycle.
- *Post-preparative stability* of the processed samples was evaluated. Triplicate LQC, MQC and HQC samples were processed, analyzed and left in the autosampler at 25°C for one day.
- *Long-term stability* was studied. Triplicate LQC, MQC and HQC samples were stored at −86°C and analyzed at the end of the study (20 days).
- *Stock solutions stability* (0.1 mg/mL of each analyte and IS) was evaluated after 30 days of storage in a refrigerator (2°C). The stored solutions were diluted to obtain the same concentration levels as the short-term stability assay.

Under all of the conditions described above, the stability was evaluated by comparing the mean recovery of the analytes and IS obtained from stored samples with the mean values obtained from freshly prepared sample, at the same concentration levels.

2.6 | Application to pharmacokinetic study

After being fully validated, the proposed method was applied for the quantitation of PAR, CXZ and ACL in plasma samples of six healthy volunteers under fasting conditions. The volunteers received a single oral dose of Dolokind-MR® tablet containing 325 mg PAR, 250 mg CXZ and 100 mg ACL. Blood samples of 5 mL were withdrawn from each volunteer and transferred into amber tubes according to the following time schedule: 0.0, 0.08, 0.16, 0.33, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0 and 12.0 h after drug administration, using ethylenediaminetetraacetic acid as anticoagulant. The samples were centrifuged immediately at 3500 rpm for 10 min and the plasma samples were labeled and kept frozen at −86°C until analysis.

3 | RESULTS AND DISCUSSION

3.1 | Method development

Optimization of sample preparation is an essential step for the best determination of the studied drugs in human plasma. Thus, various techniques were tried for sample preparation either by protein precipitation (PPT) with methanol and acetonitrile at different media (acidic, neutral and alkaline) or by liquid–liquid extraction (LLE) by ethyl acetate, tertiary butyl methyl ether, dichloromethane or diethyl ether also in different media (acidic, neutral and alkaline). The best recovery for the three drugs, best correlation coefficient and least matrix effect were attained using 3 mL tertiary butyl methyl ether as extracting solvent in acidic medium using 50 μL of 25% acetic acid (Figure 2).

Optimum extraction of the studied drugs was achieved in acidic medium. This may be explained according to their chemical structures, which contain weakly acidic groups so acidic medium suppresses their ionization and the studied drugs are thus present in the molecular nonpolar form which is easily extracted by the extracting solvent.

The mass spectrometric parameters as well as the chromatographic conditions were carefully studied and optimized as summarized in Table 2. With respect to the mass spectrometric parameters, 20 ng/mL neat solutions were infused into the mass spectrometer in order to adjust both the precursor ions and product ions. The ions were scanned in a mass range of 100–600 *m/z*. PAR and IS were easily protonated under the utilized chromatographic conditions while CXZ and ACL were easily deprotonated, thus, the mass spectrometer was operated in both positive and negative polarity modes utilizing ESI technique, resulting in an ideal intensity for the analytes' precursor ions and product ions.

The Q1 full-scan mass spectra of PAR and IS showed the protonated molecular ions $[M + H]^+$ 152.3 and 559.4, respectively, and CXZ and ACL showed the deprotonated molecular ions $[M - H]^-$ 167.9 and 353.2, respectively. Characteristic ions were produced in the Q2 as a result of the use of sufficient collision activated dissociation gas and collision energy, where the following MS/MS transitions were chosen: PAR, *m/z* 152.3 → 110.0; CXZ, *m/z* 167.9 → 131.9; ACL, *m/z* 353.2 → 75.1; and ATR (IS), *m/z* 559.4 → 440.3, as demonstrated in Figure 3. Additionally, optimization of both the capillary temperature and sheath gas flow was carried out as they can significantly alter the sensitivity. Thus, the intensity of the analytes was improved upon adjustment of capillary temperature to 270°C and sheath gas to 10 psi. However, slight changes in ion spray voltage did not show any recognizable effect on the signal intensity and it was maintained at 5500 V for positive mode and − 4500 V for the negative one.

Regarding the optimization of the chromatographic conditions, several parameters were examined including organic modifier, pH of the aqueous solution, organic modifier–aqueous ratio and column. It was obvious that the composition of the mobile phase had a noteworthy influence on the separation selectivity and sensitivity of the method. Acetonitrile and methanol were utilized as the organic modifier along with several mobile phase additives such as 0.2% aqueous formic acid, ammonium formate and ammonium acetate with pH values ranging from 3 to 7.0. The best sensitivity was achieved with the use of acetonitrile–10 mM ammonium formate pH 3.0. Moreover, various ratios of the mobile phase were also studied where acetonitrile–10 mM ammonium formate pH 3.0 (65: 35 *v*/v) was chosen. The described conditions resulted in the optimum peak shape, peak intensity and peak resolution; additionally, all the analytes were well eluted within 2.0 min as demonstrated in Figure 4. The reproducibility of retention times for the analytes was accepted for 50 injections on the same column. Separation was examined on Zorbax SB C_{18} (5 μm, 4.6 × 50 mm) and Bio Basic-8 columns for the highest chromatographic

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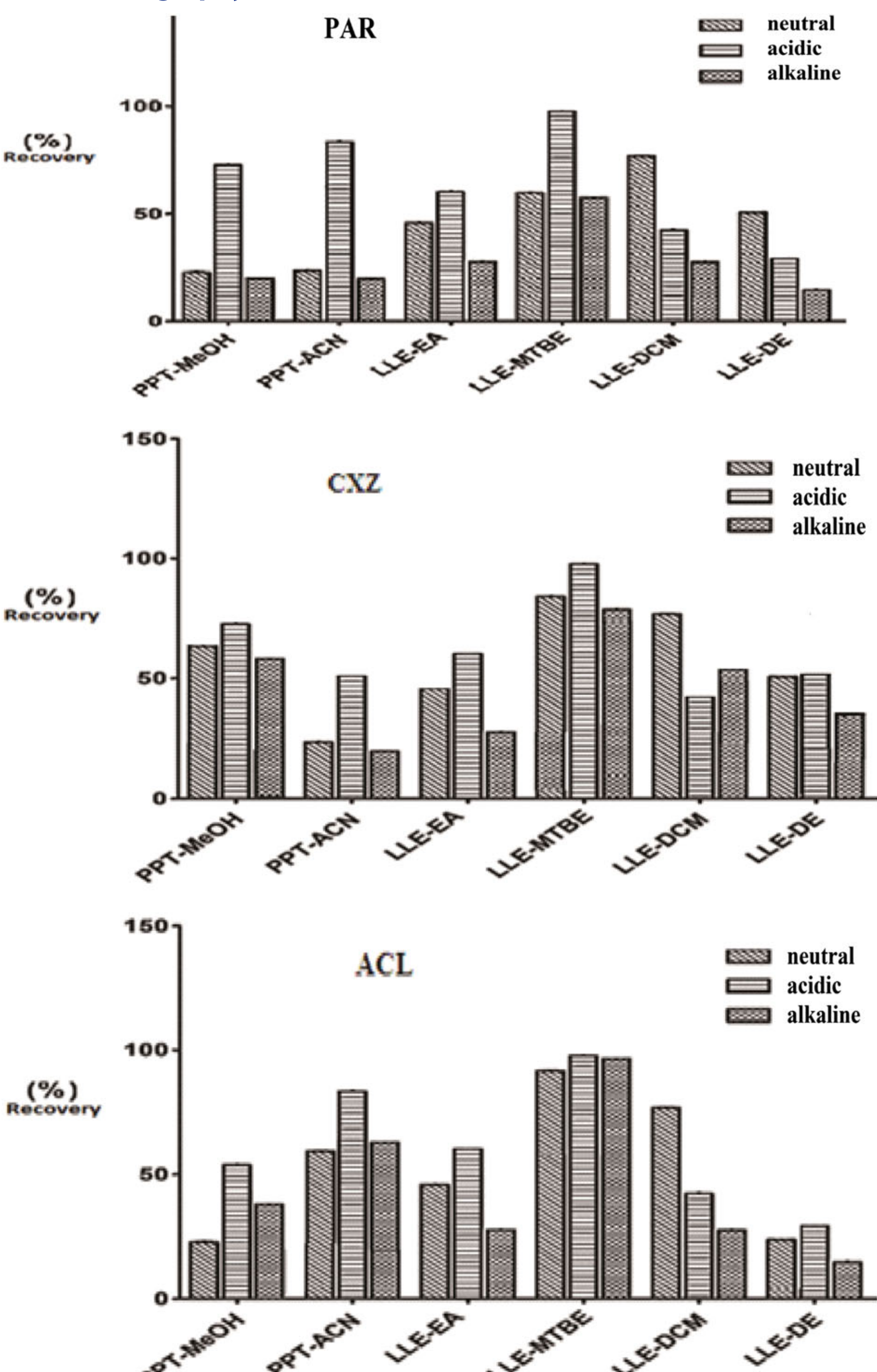

FIGURE 2 Extraction recovery of 15.0 µg/mL of each of PAR, CXZ and ACL in human plasma using different sample preparation procedures in different media

TABLE 2 LC–MS/MS parameters selected for determination of PAR, CXZ, ACL and ATR (IS)

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Declustering potential (V)	Entrance potential (V)	Collision energy (V)	Cell exit potential (V)
PAR	152.3	110.0	65	10	21	6.4
CXZ	167.9	131.9	−72.8	−10	−28.5	−5
ACL	353.2	75.1	−34	−10	−22	−11
ATR(IS)	559.4	440.3	70	10	31	30

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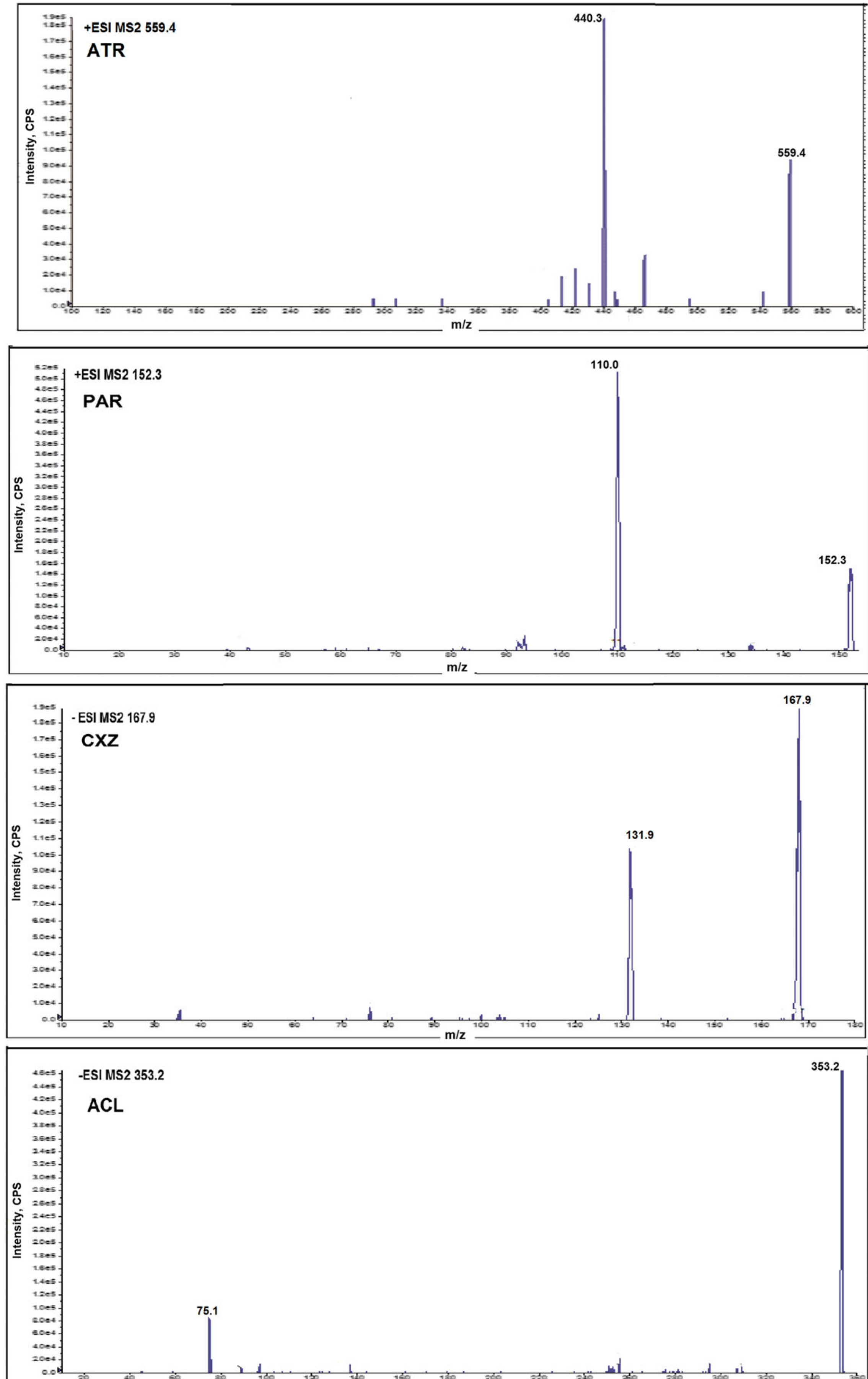

FIGURE 3 Product ion spectra of $[M+H]^+$ of atorvastatin (ATR) and PAR, and $[M–H]^-$ of CXZ and ACL

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FIGURE 4 Multiple reaction monitoring chromatograms of: (a) drug-free human plasma; (b) spiked plasma with 20.00 ng/mL ATR, 3000.00 ng/mL PAR, 1500.00 ng/mL CXZ and 1500.00 ng/mL ACL; and (c) plasma from a volunteer 3 h after administration of one tablet containing 325 mg PAR, 250 mg CXZ and 100 mg ACL

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TABLE 3 Intra- and interday precision and accuracy of the LC–MS/MS method for the analysis of PAR,CXZ and ACL in plasma samples

QC level	PAR Interday (n = 6 × 3) RE (%)	PAR Interday RSD (%)	PAR Intraday (n = 6) RE (%)	PAR Intraday RSD (%)	CXZ Interday (n = 6 × 3) RE (%)	CXZ Interday RSD (%)	CXZ Intraday (n = 6) RE (%)	CXZ Intraday RSD (%)	ACL Interday (n = 6 × 3) RE (%)	ACL Interday RSD (%)	ACL Intraday (n = 6) RE (%)	ACL Intraday RSD (%)
LLOQ	0.138	9.47	−4.33	9.98	5.44	8.61	0.89	8.38	1.94	9.68	2.33	12.17
LQC	5.20	6.68	2.44	8.60	6.83	5.54	4.85	5.61	3.26	6.72	5.11	6.63
MQC	−3.22	6.46	−7.88	3.41	−4.66	3.25	−3.65	3.95	3.89	6.45	0.09	7.17
HQC	−2.33	3.16	−2.01	2.84	1.18	3.98	3.75	3.12	5.90	6.61	10.97	2.25

RSD, Relative standard deviation; RE, relative error (n = 3 days, six replicates per day).

performance. Using the Zorbax column SB C_{18} (5 μm, 4.6 × 50 mm) at room temperature showed better response, even at LLOQ levels of the analytes.

Although several HPLC-MS/MS methods were developed for the determination of each of PAR, CXZ and ACL singly or in combination with other drugs in human plasma, to the best of our knowledge no method for their simultaneous determination in human plasma has been reported. Therefore, our proposed method is the first HPLC-MS/MS attempt at their simultaneous determination in human plasma. It is apparent that our method has combined most of the advantages which could be presented by an HPLC-MS/MS method. The employment of HPLC with small column size (5 μm, 4.6 × 50 mm) led to a short run time of ~2 min, in addition to good resolution owing to the small particle size. The utilization of a flow rate of 0.6 mL/min offered low levels of solvent consumption, which is considered to be cost effective. Moreover, a simple isocratic mobile phase was demonstrated and a very small injection volume (3 μL) made our proposed method more challenging.

3.2 | Method validation

A full validation process was conducted according to the US Food and Drug Administration (2013) guidelines for bioanalytical method validation.

3.2.1 | Specificity

Comparison of the chromatograms obtained from blank plasma and blank plasma spiked with PAR, CXZ and ACL at the LLOQ reflected the lack of interfering peaks from endogenous plasma components at the retention times of analytes and IS.

3.2.2 | Linearity and range

The plasma calibration curves were constructed within the ranges 0.03–30.0 μg/mL for PAR, 0.015–15.0 μg/mL for CXZ and 0.15–15.0 μg/mL for ACL, with good reproducibility and linearity. The correlation coefficients of the generated calibration curves were > 0.999.

$$y = 0.205\times + 0.00412 \quad R = 0.9991 \text{ for PAR}$$
$$y = 0.158\times + 0.00241 \quad R = 0.9994 \text{ for CXZ}$$
$$y = 0.0009\times - 0.0001 \quad R = 0.9997 \text{ for ACL}$$

The back-calculated concentrations at all points on the standard curve were within ±15% of the nominal concentrations. The lowest concentration at signal-to-noise ratios of 10 with the RSD <20% was taken as LLOQ, and was 0.03 μg/mL for PAR, 0.015 μg/mL for CXZ and 0.15 μg/mL for ACL.

3.2.3 | Precision and accuracy

Accuracy and precision were within ±20% for LLOQ and ± 15% for the other QC samples. The intraday precision (RSD) was 2.84–9.98, 3.12–8.38 and 2.25–12.17% for PAR, CXZ and ACL respectively. However, the intraday accuracy (RE) was −7.88–2.44, −3.65–4.85 and 0.09–10.97% for PAR, CXZ and ACL, respectively. The interday variability of the assay was evaluated by analyzing QC samples for three successive days. The interday precision (RSD) was 3.16–9.47, 3.25–8.61 and 6.45–9.68% for PAR, CXZ and ACL, respectively. On the other hand, the interday accuracy (RE) was −3.22–5.20, −4.66–6.83 and 1.94–5.90% for PAR, CXZ and ACL, respectively, as shown in Table 3.

3.2.4 | Recovery

The mean extraction recovery for the analyzed drugs was calculated at all QC levels and was >85%. It was 93.10–110.10% for PAR, 96.40–106.50 for CXZ and 97.33–112.24% for ACL.

TABLE 4 Results of stability tests for determination of PAR,CXZ and ACL in QC plasma samples by the proposed LC-MS\MS method

Stability condition	QC level	Precision (RSD, %) (n = 3) PAR	CXZ	ACL	Accuracy (RE, %) (n = 3) PAR	CXZ	ACL
Short-term stability	LQC	10.23	7.18	3.73	5.56	5.85	6.74
	MQC	6.78	4.39	3.05	2.00	4.44	10.55
	HQC	4.84	4.76	3.67	−5.56	−2.5	7.22
Freeze–thaw stability	LQC	10.23	6.04	3.73	5.56	5.11	6.74
	MQC	1.41	3.46	3.05	−1.41	5.00	10.55
	HQC	1.97	2.65	5.68	−3.33	1.11	6.11
Post-operative stability	LQC	0.64	1.44	2.81	8.22	3.04	2.07
	MQC	3.09	1.20	0.597	6.26	6.11	6.53
	HQC	1.47	2.12	1.59	−1.53	4.17	5
Long-term stability	LQC	3.01	4.70	4.92	−3.85	4.96	3.93
	MQC	4.71	1.56	3.69	5.70	1.94	3.33
	HQC	5.59	2.55	3.98	−5.56	2.05	−1.11
Stock solution stability	LQC	4.80	1.49	3.64	−7.18	−7.33	4.81
	MQC	2.94	4.79	3.41	6.81	−2.64	10.28
	HQC	3.43	4.52	4.69	−8.33	−3.26	6.94

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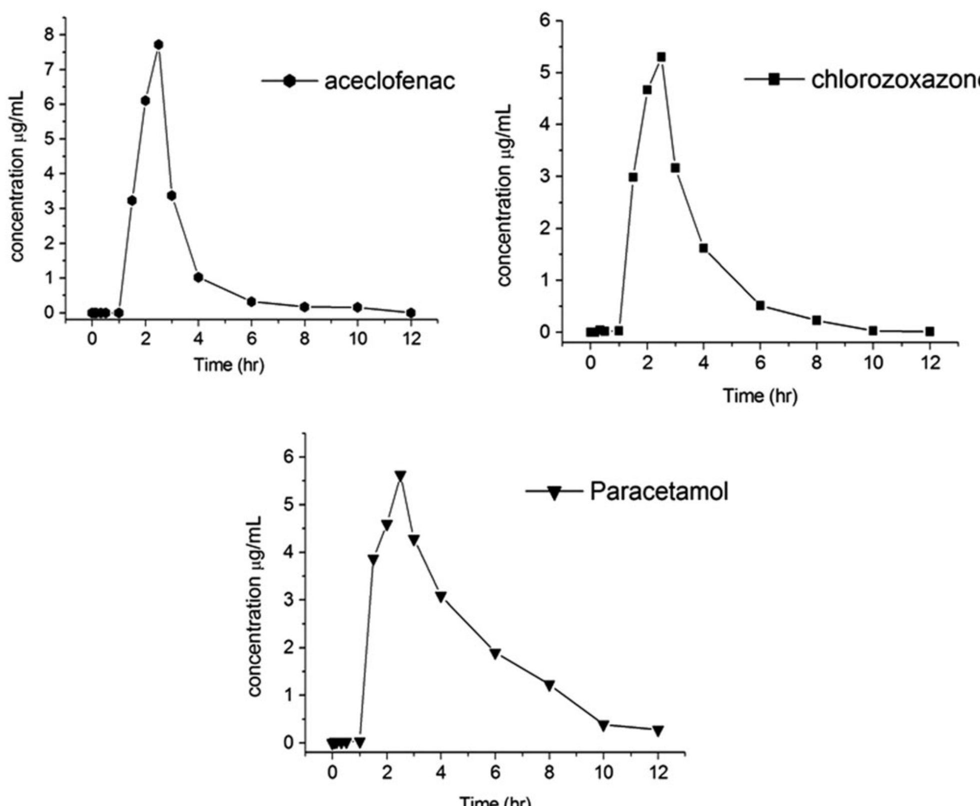

FIGURE 5 Mean plasma concentration after a single oral dose administered of one tablet containing 325 mg paracetamol, 250 mg chloroxazone and 100 mg aceclofenac

TABLE 5 Pharmacokinetic parameters of PAR, CXZ and ACL in human plasma following a single oral administration

	Parameter						
Drug name	C_{max} (μg/mL)	T_{max} (h)	AUC_{0-t} (μg h/mL)	AUC_{extra} (μg h/mL)	$AUC_{0-\infty}$ (μg h/mL)	Lz (1/h)	$T_{1/2}$ (h)
PAR	5.63	2.5	22.2315	0.862275	23.0938	0.312901	2.21523
CXZ	5.3	2.5	12.8499	0.019113	12.869	0.623202	1.11224
ACL	7.72	2.5	10.748	0.235085	10.9831	0.423286	1.63754

AUC_{0-t}, Area under the plasma concentration curve from administration to last observed concentration at time t; AUC_{extra}, area under curve extrapolated after last experimental point; $AUC_{0-\infty}$, area under the plasma concentration curve extrapolated to infinite time; Lz, terminal elimination rate constant; C_{max}, plasma maximum concentration; T_{max}, time of the maximum plasma concentration; $T_{1/2}$, plasma concentration half-time.

3.2.5 | Matrix effect

It was found that the RSD of peak area ratios (analyte/IS), was <2% and the RSD of peak areas of individual compounds was <4%. Therefore the absence of a relative matrix effect on ionization (suppression or enhancement) for the developed method was ascertained.

3.2.6 | Stability

For the different stability experiments (short-term stability, freeze–thaw stability, post-preparative stability, long-term stability and stock solution stability), three sets of low, medium and high plasma QC samples were prepared and processed as explained in Section 2.5.6. Under all of the studied conditions, the samples were considered to be stable as there was no significant difference observed for each concentration compared with the nominal concentration (Table 4).

3.3 | Application to real plasma samples

The validated method was successfully applied to the pharmacokinetic study of the combined drugs PAR, CXZ and ACL in human plasma of healthy volunteers. All volunteers were fasting males with an average weight of 70 kg, average height of 166 and body mass index of 26.13. Figure 5 shows the mean plasma concentration vs time curves for the studied drugs and the obtained results (Table 5) were in good agreement with the pharmacokinetic profile of PAR (Borin & Ayres, 1989), CXZ (Frye & Stiff, 1996) and ACL (Najib et al., 2004) concentrations in human plasma.

4 | CONCLUSION

A rapid, selective and sensitive HPLC-MS/MS method was developed and validated for the simultaneous determination of paracetamol,

chlorzoxazone and aceclofenac in human plasma. The major advantage of the proposed method is the analysis of the studied drugs on a single chromatographic system with great sensitivity, low solvent consumption, very small injection volume and short run-time. The results of the proposed method revealed that it could be applied where limited resources are available for the detection and quantitation of analytes even in nanogram concentrations, like in human plasma. The method was successfully applied to determine paracetamol, chlorzoxazone and aceclofenac plasma concentrations in a pharmacokinetic study involving healthy Egyptian volunteers.

ORCID

Dalia Mohamed http://orcid.org/0000-0002-3954-7930
Mona S. Elshahed http://orcid.org/0000-0002-5815-6556

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