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Research Article

Simultaneous determination of salbutamol and clenbuterol in human plasma using liquid chromatography coupled to tandem mass spectrometry

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Abstract

Salbutamol (SAL) and clenbuterol (CLEN) are β-agonist drugs which are illegally abused in animal feeds in an attempt to promote animal growth and to increase muscle weight. In this study, LC-MS/MS method was developed and validated to simultanously determine plasma SAL and CLEN concentrations in order to contribute to assess the toxicity and optimize the treatment efficacy of these drugs. SAL, CLEN and SAL-d3, CLEN-d9 as the internal standards were extracted from plasma samples with methanol. 10 µL of sample was injected into Inertsil® ODS-3V column C18 (150×4.6 mm, 5µm) on a HPLC system coupled to AB Sciex Triple Quad 5500 tandem mass spectrometer. The mobile phase consisted of a mixture of acetonitrile and 5mM ammonium acetate under a flow rate of 1 ml/min. The multiple reaction monitoring transitions used for identification and quantification were *m/z* 240→166 and *m/z* 240→148, respectively for SAL, and *m/z* 277→168 and *m/z* 277→203, respectively for CLEN, *m/z* 244→151 for SAL-d3 and *m/z* 286→204 for CLEN-d9, in positive ESI mode. The total run time was 15 min. The retention times for SAL and CLEN were 4.7 and 5.6 min, respectively. Linearity was in the range from 0.15-24 ppb for SAL and from 0.05-8 ppb for CLEN. Mean intra-day and inter-day precision levels for SAL and CLEN were above 90%. LLOQ of SAL and CLEN were 0.15 and 0.05 ppb, respectively. The stability study displayed SAL and CLEN degradation below 11% after 24 hours at RT. This analytical method could be used to clinical trials and assess the toxicity of these drugs.

1. INTRODUCTION

β-agonists are compounds derived from catecholamines (adrenaline, noradrenaline and dopamine). They bind to β-receptors located on different cell types including neurons, muscles, adipose tissue, and blood. Today, three types of β receptors have been identified in which β1 receptors are mainly found in the heart, kidney, β2 receptors are located in the smooth muscle of the bronchi, the uterine muscle, smooth muscle of blood vessels, muscles of the bones, liver, pancreas and glands and β3 receptors are located in fatty tissues and digestive tract. The effects on the β2 receptor result in smooth muscle (blood vessels and internal organs) relaxation and stimulating insulin release as well as glycogen degradation. Therefore, β-agonists

are commonly used in the treatment of asthma and bronchial diseases in humans, two of which are salbutamol (SAL) and clenbuterol (CLEN) [1].

However, these drugs were sometimes illegally added to animal feed to decrease fat under the skin, and increase muscle and weight, especially in terms of accelerating growth of animal. Because β-agonists are often strong stimulants and cause hepatic function impairment, these drugs are harmful to animals and also to consumers eating β-agonist residues-contaminated animal meats [2-5].

In Vietnam, SAL and CLEN are prohibited in the livestock by the Ministry of Agriculture and Rural Development. However, these substances are still abused in animal breeding by a number of livestock farmers. The presence of SAL and CLEN in cattle and poultry feed has been qualitatively and quantitatively determined at high levels. SAL and CLEN accumulation in food of animal origin will affect the consumer's health through the food chain, including symptoms of arrhythmia, tachycardia, hypertension, bronchospasm, edema, tremor, muscle paralysis, dizziness, etc [2-3]. Determination of SAL and CLEN levels in human plasma contributes to evaluate the toxicity of these substances. Currently, there are a number of analytical methods to measure SAL and CLEN concentrations such as capillary electrophoresis method, high performance liquid chromatography (HPLC) equipped with either ultraviolet (UV) or PDA (photodiode array) or mass-spectrometric (MS) detectors, … During this last decade, LC-MS/MS appeared in many large clinical laboratories, providing both higher specificity and sensitivity. LC-MS/MS reaches sufficient sensitivity to quantify SAL and CLEN plasma levels, or even residue levels [8-14]. The aim of this study is the development and validation of a sensitive and reliable analytical method to quantify SAL and CLEN levels in human plasma.

2. MATERIALS AND METHODS

2.1. Chemicals, reagents and plasma from healthy volunteers

Salbutamol sulfate (SAL), clenbuterol hydrochloride (CLEN) and SAL-d3, CLEN -d9 were the gifts from Sigma-Aldrich (Ho Chi Minh city, Vietnam). Acetonitrile (ACN) and methanol for ultra-performance liquid chromatography/mass spectrometry, ammonium hydroxide solution and formic acid were obtained from Merck (Ho Chi Minh city, Vietnam). Human plasma samples for analytical development were obtained from healthy volunteers in Cho Ray hospital (Ho Chi Minh city, Vietnam). The local Ethics Committee has approved the blood sampling for this purpose.

2.2. Preparation of reagents, stock solutions, calibrations and quality control (QC) samples

2.2.1. Preparation of reagents

Solutions of 2% formic acid and 5% methanol were prepared by diluting the concentrated solutions with double distilled water. The 5% ammonium in methanol was obtained by diluting the 5 ml of 25% ammonium hydroxide solution in 95 ml of absolute methanol.

2.2.2. Preparation of stock solutions

Separate stock solutions containing 1 mg/ml SAL, CLEN, SAL-d3 and CLEN-d9 were prepared in absolute acetonitrile. The working solutions of 1 µg/ml SAL and CLEN were obtained by diluting the primary stock solutions with acetonitrile. 1µg/ml SAL-d3 and CLEN-d9 were prepared in absolute acetonitrile from stock solutions. These stock solutions were stored at -20 °C.

2.2.3. Preparation of calibrations and quality control (QC) samples

The SAL and CLEN calibration and quality control (QC) samples were separately prepared from 1 µg/ml SAL and CLEN stock solutions into blank plasma to achieve calibration concentrations range from 0.15, 0.75, 0.3, 3, 12, 24 ng/ml for SAL and 0.05, 0.1, 0.25, 1, 4, 8 ng/ml for CLEN, and to achieve QC concentrations of 0.3, 9, 18 ng/ml and 0.1, 3, 6 ng/ml for SAL and CLEN, respectively. Internal standard concentrations were prepared in calibrations and QC samples at 20 and 1 ng/ml for sal-d3 and clen-d9, respectively. These samples were then processed similarly to the real samples.

2.2.4. SAL and CLEN extraction from plasma samples

900 µl of plasma, 900 µl of 5% NH_4OH and 200 µl of mixture containing 20 ng/ml sal-d3 and 1 ng/ml clen-d9 as internal standards were added to a 5ml eppendorf tube. SAL and CLEN

extraction were performed by vortexing for 30 min at room temperature (RT). The sample was then centrifuged at 13,440 *xg* for 10 min. 1.5 mililiters of supernatant were transferred into OASIS HLB cartridge 1ml (Waters, USA) that was previously conditioned by subsequent washing with 1 ml of pure methanol and 1 ml of water. Next, washing SPE column was assayed either with 1 ml of pure water or 1 ml of 5% methanol. Then, SAL, CLEN and internal standards simultaneous extraction was examined by adding either 1.5 ml of pure methanol or 1.5 ml of 5% ammonium in methanol. The solution eluted from cartridge was collected into a 2-ml eppendorf and evaporated under a nitrogen flow to dryness at 65 °C. The residues were reconstituted with 200 µL of ACN (vortexed for 1 min). After centrifugation at 13,440 *xg* for 5 min at room temperature, the supernatants were transferred into vials for the liquid chromatography–tandem mass spectrometric (LC-MS/MS) analysis.

2.3. Instrumentation and MS assay

Chromatography: Chromatographic separation of the extracted samples was obtained using a Inertsil® ODS-3V C18 column (150mm x 4.6 mm, 5µm particle size) (GL sciences). Ten microliters of the reconstituted extract were injected onto the column maintained at 25 °C on a HPLC system (Shimazdu) coupled to AB Sciex Triple Quad 5500 tandem mass-spectrometer (LabX). The tray temperature in the autosampler was kept at 4 °C. The mobile phase consisted of solvent A (acetonitrile containing 0.1% formic acid) and solvent B (aqueous solution containing 5 mmol/L ammonium acetate and 0.1% formic acid). Mixture of solvents A and B was employed as a gradient with a flow rate of 1 ml/min for SAL and CLEN analysis. Over 0 –0.01, solvent A was kept constant at 5%. Then over 0.01 –6 minutes, solvent A linearly increased from 5% to 100% and returned to 5% over 1.0 min, and finally equilibrated for 8.0 min before next run. The total run time was 15.0 min.

LC-MS/MS system: Simultaneous quantification of SAL and CLEN was performed by LC-MS/MS system that consists of AB Sciex Triple Quad 5500 micro-tandem mass spectrometer fitted with a Z-spray ion source. The instrument was operated in an electrospray positive ionization (ESI) mode. Ionization was achieved in the positive ion mode using the following settings: capillary voltage 3.0 kV, desolvation temperature 650 °C, collision energy set to 26/20/22V for SAL_{148}/SAL_{166}/SAL-d3 and 38/21/23V for $CLEN_{168}$/$CLEN_{203}$/CLEN-d9. SAL and CLEN were monitored by detecting specific product ions, resulting from the fragmentation of their precursor ions using multiple reaction monitoring (MRM) mode: SAL $[M+H]^+$, *m/z* 240→166 and *m/z* 240→148 and CLEN $[M+H]^+$, *m/z* 277→168 and *m/z* 277→203. All fragments were used for SAL and CLEN qualification, in which the transitions *m/z* 240→148 and *m/z* 277→203 were used for SAL and CLEN quantification, respectively. The transitions *m/z* 244→151 and *m/z* 286→204 were used for SAL-d3 and CLEN-d9 quantification, respectively with a dwell time of 100 ms on each mass. All LC-MS/MS instrument parameters were presented in table 1.

Table 1. Multiple reaction monitoring (MRM) parameters for LC-MS/MS

Analytes	Ionisation mode	Parent ions (m/z)	Product ions (m/z)	Collision energy (V)	Dwell time (ms)
Salbutamol	Positive	240	148	26	100
	Positive		166	20	100
Salbutamol-d3	Positive	243	151	22	100
Clenbuterol	Positive	277	203	21	100
	Positive		168	38	100
Clenbuterol-d9	Positive	286	204	23	100

2.4. Plasma SAL and CLEN LC-MS/MS assay validation

The assay was fully validated according to the acceptance criteria published by US Food and Drug Administration 2001 and European Medicines Agency 2011[6,7]. All statistics were performed using JMP software 10 (JMP software; SAS Institute, Cary, NC).

2.5. Linearity

To evaluate linearity of the method, the calibration standards were separately prepared and analyzed in replicate of 3 in 3 separate analytical runs. The curves were considered linear if the coefficients of determination R^2 were >0.99 (as calculated by non-weighted linear regression) and the coefficient of variation (CV) from their nominal concentrations should be within ± 20% for lower limit of quantification (LLOQ) and within ± 15% at the other levels. Calibration curves were based on the peak area ratios of SAL_{148}/SAL-d3 or $CLEN_{203}$/CLEN-d9 versus SAL_{148} or $CLEN_{203}$ concentrations, respectively. The regression curves were then used to calculate plasma SAL or CLEN levels.

2.6. Accuracy and precision

The accuracy and precision validation were carried out by analyzing the samples at 3 QC levels in replicate of five. Intra and inter-assay accuracies were calculated from the differences between the nominal and the observed concentrations. The within-run CV% value of precision and accuracy should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%.

2.7. Recovery

Recovery of plasma SAL and CLEN concentrations was assessed by comparing the analytical results for extracted samples at three concentrations (0.3, 9, 18 ng/ml for SAL and 0.1, 3, 6 ng/ml for CLEN) with unextracted standards that represent 100% recovery. For each QC concentration level, 6 blank plasma samples were spiked with SAL and CLEN and internal standards, and then were extracted as previously described procedure.

2.8. Selectivity

Method selectivity were performed by analyzing blank plasma samples, and plasma samples collected from 6 different healthy volunteers and spiked SAL, CLEN and IS to differentiate the analytes from endogenous components in the matrix or other components in the sample. Absence of interfering components is accepted where the response is less than 15% of QC levels of SAL and CLEN and 5% for IS [6].

2.9. Lower limits of Quantification (LLOQ)

The LLOQ is considered being the lowest calibration standard at which SAL and CLEN can be quantified reliably, with an acceptable accuracy and precision. In addition, the analyte signal of the LLOQ sample should be at least 5 times the signal of a blank sample.

2.10. Matrix effect

Matrix effects were assessed by analyzing six lots of plasma matrix collected from healthy volunteers and spiked SAL and CLEN at QC concentrations (0.3, 9, 18 ng/ml for SAL and 0.1, 3, 6 ng/ml for CLEN). One mililiter of pure solution containing SAL and CLEN and IS in ACN at different concentrations was added post-extraction residues. Pure solutions and reconstituted samples were injected into the LC-MS/MS system using previously described chromatographic conditions. The variability of difference between the analytical post-extraction results and the pure standards had to be <15%.

2.11. Stability Study

The stability of SAL and CLEN in plasma was investigated at low and high concentrations (0.3, 18 ng/ml for SAL and 0.1, 6 ng/ml for CLEN) at RT for 24 hours before extraction by the comparison with freshly prepared samples. Additionally, the stability of SAL and CLEN and IS in samples processed and stored in the autosampler (4 °C) was determined after 24 hours. Analytes were considered stable in plasma if the determined concentrations did not deviate more than ± 15% from the concentration determined at time zero. Testing stability was performed in three replicates at each concentration level.

3. RESULTS

3.1. SAL and CLEN extraction from plasma

SAL and CLEN extraction from plasma was performed by using SPE. After passing the samples at flow rate onto cartridge, SPE was washed once with 1 ml of 5% methanol and then eluted once with 1.5 ml of pure methanol. SAL and CLEN signals were detected with a high intensity by LC-MS/MS system, suggesting that the washing and eluting steps were sufficient to remove the impurities from SAL and CLEN in plasma.

3.2. Chromatographic results

The total run time for the analysis of one sample was 15.0 min, including pre-run and post-run. Retention times were 4.6 min for SAL and 5.6 for CLEN. All signals were clearly separated and well shape. No interfering peak was detected at the *m/z* transitions and retention times of interests. Typical ion chromatograms of extracts from blank plasma sample, plasma reconstituted with SAL (9 ppb) and CLEN (3 ppb) and IS, standard solutions of SAL (9 ppb) and CLEN (3 ppb), and internal standard solutions of SAL-d3 (2 ppb) and CLEN-d9 (1 ppb) are shown in Fig. 1, 2, 3 and 4, respectively.

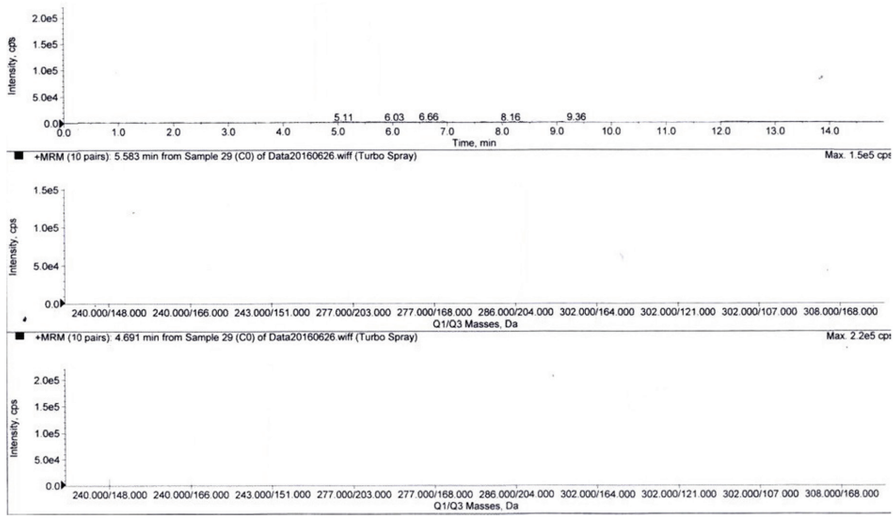

Figure 1. Typical ion chromatograms of extracts from blank plasma sample

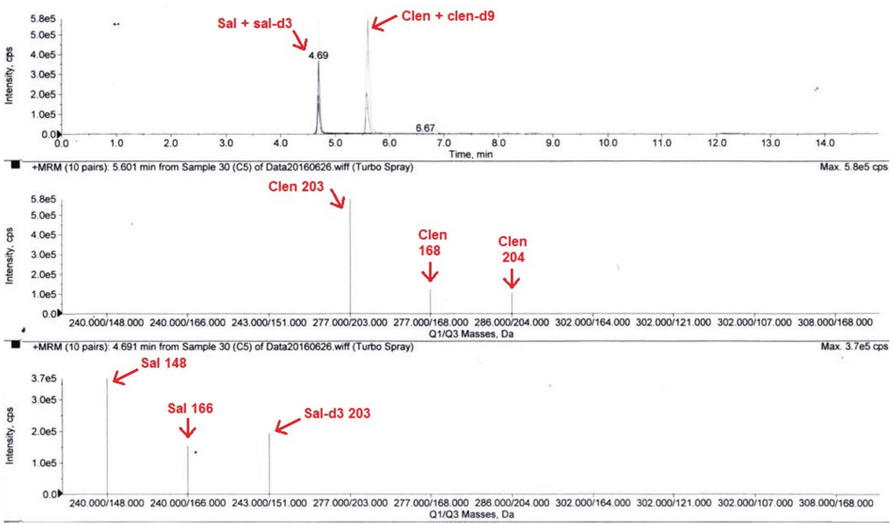

Figure 2. Typical ion chromatograms of extracts from plasma sample fortified with SAL (9 ppb), CLEN (3 ppb), SAL-d3 (2 ppb) and CLEN-d9 (1 ppb)

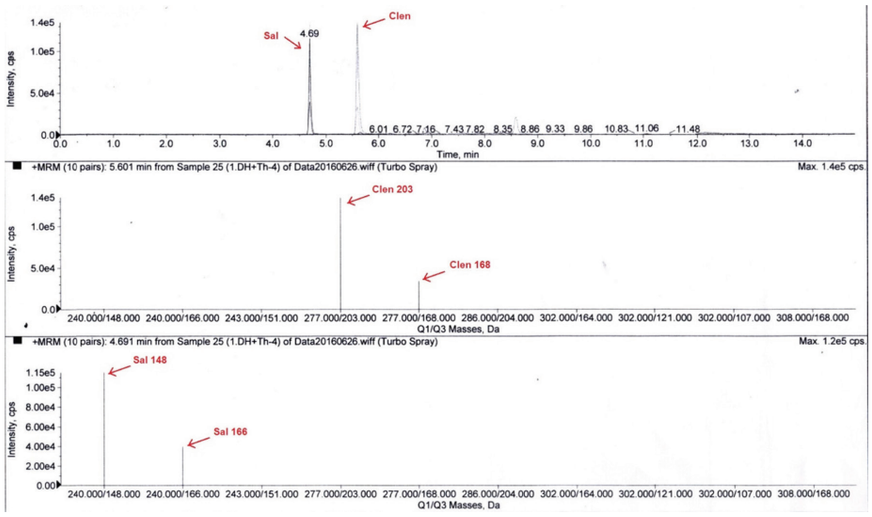

Figure 3. Typical ion chromatograms of standards of SAL (9 ppb) and CLEN (3 ppb)

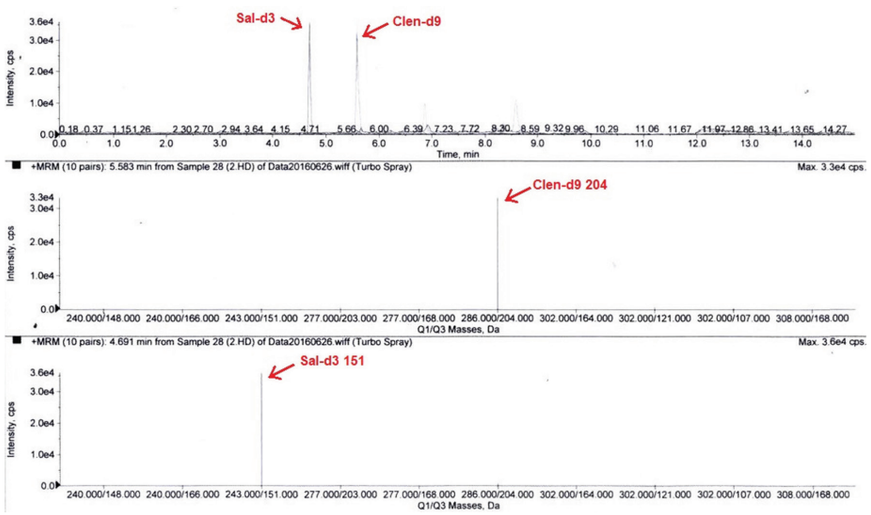

Figure 4. Typical ion chromatograms of internal standards of SAL-d3 (2 ppb) and CLEN-d9 (1 ppb)

3.3. Validation results

The calibration curves of SAL and CLEN were linear over the concentration range from 0.15 to 24 ng/ml and from 0.05 to 8 ng/ml, respectively. The coefficients of determination (R^2) of these curves ranged from 0.996-0.999 for SAL and CLEN, with a mean value of 0.997 ± 0.001 ($p < 0.0001$). Limits of quantification were found at 0.15 ng/ml (CV%, 9.22%) and 0.05 ng/ml (CV%, 3.21%) for SAL and CLEN, respectively. Average recoveries at the low, medium, and high QC concentrations in plasma samples were > 72% for SAL and CLEN (CV%, from 5.20% - 7.41% for SAL and from 6.65% - 12.42% for CLEN) (see Table 2). The inter-day and intra-day accuracies were in the range from 90-106% at the low, medium, and high QC concentrations for SAL and CLEN. Inter-day and intra-day precision levels were > 90% at all SAL and CLEN QC concentrations, (CV%, from 2.96% - 7.39% for SAL and from 6.93% - 8.97% for CLEN). All inter- and intra-day assays are summarized in Tables 3. Matrix effects and ion suppression were less than 6%. The results of this experiment suggest that matrix effects have a minimal influence on the results of method. No signal of analytes in ACN was observed after injecting a highest calibrator concentration (24 ng/ml for SAL and 8 ng/ml for CLEN). The stability

study did not significantly display SAL and CLEN degradation after 24 hours at room temperature (see Table 4). In the post-processing stability study, no significant degradation has been detected in the samples (4 °C) left in the autosampler for 24 hours.

Table 2. Absolute recovery of the developed extraction method for salbutamol and clenbuterol

Analyte	QC Sample	A	B	Recovery (%)
SAL	LQC	0.216	0.3	72.07
	MQC	6.47	9	72.11
	HQC	13.0	18	72.26
CLEN	LQC	0.08	0.1	77.01
	MQC	2.18	3	72.53
	HQC	4.50	6	75.00

Table 3. Precision and accuracy of the quality control samples of salbutamol and clenbuterol at low, medium and high QC concentrations

Analyte	QC Sample	Accuracy		Precision	
		% Intra-day (n=15)	% Inter-day (n=5)	% Intra-day (n=15)	% Inter-day (n=15)
SAL	LQC (0.3 ng/ml)	98.31	97.63	97.04	92.61
	MQC (9 ng/ml)	93.25	94.94	95.46	92.99
	HQC (18 ng/ml)	90.51	91.77	95.03	90.87
CLEN	LQC (0.1 ng/ml)	106.17	106.84	91.30	94.57
	MQC (3 ng/ml)	103.93	104.08	93.07	94.23
	HQC (6 ng/ml)	104.81	98.41	93.05	92.48

Table 4. Stability of SAL and CLEN at RT for 24 hours (n=3)

Analyte	Concentration at T0	Concentration at T24 (RT)	Post-processing concentration at T24 (4 0C)	% Change (a)	% Change (b)
SAL (0.3ng/ml)	0.30	0.27	0.26	10.0	3.70
SAL (18ng/ml)	18.59	16.56	6.49	10.91	0.42
CLEN (0.1ng/ml)	0.1021	0.1007	0.1025	1.37	1.78
CLEN (6ng/ml)	6.00	5.85	5.55	2.50	0.53

4. DISCUSSION AND CONCLUSION

Simultaneous determination of SAL and CLEN concentrations in human plasma may contributes to evaluate the contamination degrees from food and avoid the toxicity of these substances. To date several quantitative methods have been developed to measure the β2-agonist concentrations in food and biological matrix [8-14]. However, little is known about the potential interest to determine SAL and CLEN in plasma where their concentration is supposed to correlate to the pharmacological effects.

In our study, this method was shown to be highly selective with no interference or environment contamination presenting in blank plasma samples for SAL and CLEN, and internal standard retention times.

We established SAL and CLEN extraction procedure from plasma was performed by using SPE. Removing the impurities in plasma on SPE column with 1 ml of 5% methanol improved SAL and CLEN qualification and quantification using LC-MS/MS system.

The total run time was 15 min, in which 8 minutes were spent to equilibrate LC-MS/MS conditions before next run. So the retention times for SAL and CLEN were stable at 4.7 and 5.6 min, respectively in each run. Linearity was in the range from 0.15-24 ppb for SAL and from 0.05-8 ppb for CLEN. Mean intra-day and inter-day precision levels for SAL and CLEN were above 90%. LLOQ of SAL and CLEN were 0.15 and 0.05 ppb, respectively. The stability study displayed SAL and CLEN degradation below 11% after 24 hours at RT. Moreover, this method presented sensitivity at LLOQ level of 0.15 ng/ml for SAL and 0.05 ng/ml for CLEN. The IS used in previous method was the acetaminophen, which could possibly be used as concomitant therapy in some patients [15]. Most likely, the use of the SAL-d3 and CLEN-d9, as IS, contributed to the improved performances.

In conclusion, a robust, reliable and rapid analytical method was developed for determination of SAL and CLEN concentrations in human plasma. This analytical method could be used to quantify plasma SAL and CLEN concentrations in poisoning patients and patients under these drugs therapy.

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Conflict of interest

There are no conflicts of interest.

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Ethical approval

This study was approved by the Ethics Committee of University of Pharmacy and Medicine at Ho Chi Minh City.

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