

LC-MS/MS Method for Quantitation of Hydrochlorothiazide and Nifedipine in Human Plasma

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Abstract

We have developed and validated a novel, sensitive, selective and reproducible reversed-phase high-performance liquid chromatography method coupled with electrospray ionization mass spectrometry (HPLC-ESI-MS/MS) for the simultaneous quantitation of hydrochlorothiazide (HCTZ) and nifedipine (NFP) from only 200 μ L of human plasma. Diazepam (DZP) was used as an internal standard (IS). The analytes were extracted by a liquid-liquid extraction procedure with ethyl acetate-dichloromethane and separated on a reversed-phase Polaris 5 C18-Analytical column using a mobile phase composed of methanol containing 0.1% (v/v) formic acid and 5mM aqueous ammonium formate pH 6.0, delivered at a flow-rate of 300 μ L/min. Multiple reaction monitoring was performed in the negative ion mode using the transition m/z 296.1 \rightarrow m/z 205.2 (HCTZ), positive ion mode transitions m/z 347.2 \rightarrow m/z 315.1 (NFP) and m/z 285.0 \rightarrow m/z 193.2 (DZP) to quantify the drugs. Calibration curves in spiked plasma were linear ($r^2 \geq 0.9953$) from 5-2000 ng/mL for HCTZ and 5 - 400 ng/mL for NFP with a lower limit of quantification (LLOQ) of 5 ng/mL for both drugs. The intra- and inter- assay precisions (coefficient of variation) were less than 9% and the mean extraction recoveries were 98.1% (HCTZ), 99.5% (NFP) and 93.8% for the IS (DZP). The validated method was successfully applied to a limited population pharmacokinetics study of HCTZ and NFP among patients on hypertension care.

Keywords

LC-MS/MS; Hydrochlorothiazide; Nifedipine; Hypertension; Human plasma; Non-communicable diseases.

1. INTRODUCTION

Hypertension has been, and remains a significant health problem in many developing countries experiencing epidemiological transition from communicable to non-communicable diseases.

Clinical therapeutic studies of non-communicable diseases such as hypertension has interested the practitioners and academicians alike due to intricacies and limited data among the Sub-Saharan Africa populations (Dipanjan *et al.*, 2017). Monotherapy is also becoming increasingly ineffective at bringing the desired treatment outcomes due to the multifactorial etiology of hypertension, most practitioners have therefore resorted to combination therapy involving different classes of antihypertensive agents being administered as either fixed dose combination formulary or co-administered separate formulations as per recommendations of the European hypertension guidelines (Volpe *et al.*, 2012; Takao *et al.*, 2007). The rationale of this strategy is partly based on the different effects on the renin angiotensin system caused by the various antihypertensive agents, thus improving antihypertensive effect (Aram V. *et al.*, 2003; Alan H., 2012).

Hydrochlorothiazide (6-chloro-3, 4-dihydro-2H-1, 2, 4-benzothiadiazine-7-sulfo-namide-1, 1-dioxide, HCTZ) (Figure 1A), is a diuretic and antihypertensive agent that reduces plasma volume by increasing the excretion of sodium, chloride and water and, to a lesser extent, that of potassium as well (Lant A., 1985). Hydrochlorothiazide is one of the oldest thiazide diuretics, often prescribed in combination with other antihypertensive drugs such as beta blockers, angiotensin-converting enzyme inhibitors, or angiotensin II receptor blockers (Trenkwalder P., 2002). Nifedipine, 1, 4-dihydro-2, 6-dimethyl-4-(2-nitrophenyl)-3, 5- pyridine dicarboxylic acid dimethyl ester (Figure 1B), is a dihydropyridine calcium channel blocker used widely in the treatment of cardiovascular disorders, such as hypertension, angina and atherosclerosis (Itatani M., 1985). Nifedipine acts by inhibiting the transmembrane influx of calcium into cardiac and vascular smooth muscle cells, thus reducing muscle contraction and has predominantly vasodilatory effects on arteries with minimal effects on the myocardium and cardiac conduction (Danser Ah., 1995). Nifedipine is also known for its low bioavailability mainly due to pre systemic metabolism, which may result in very low plasma concentration and substantial inter subject pharmacokinetic variability (Wuffle *et al.*, 1984). Moreover, the most undesirable characteristic of nifedipine is its high photochemical sensitivity. A 96% degradation has been observed when methanolic solution of nifedipine is exposed to laboratory light for 2 h (Sankar S.G *et al.*, 1987). These unfavorable pharmacokinetics and physical characteristic make the determination of nifedipine in plasma tricky.

Several methods have been reported for quantification of HCTZ and NFP individually (Ramakrishna N. *et al.*, 2005; Wang *et al.*, 2011; Shah H. *et al.*, 2009; Hisham S *et al.*, 2000; Yunzhen G. *et al.*, 2007), or simultaneously with other antihypertensive agents (Olga S. *et al.*, 2014; Xiangjun *et al.*, 2014; Ramkumar *et al.*, 2014; Jaivik V. *et al.*, 2017; Essam E *et al.*, 2014) in human plasma.

Hisham S. *et al.*, 2000; developed a method for quantitation of nifedipine in human plasma, the LC-UV method was limited in quantifying lower levels of the drug in a biological matrix due to its low sensitivity and poor selectivity. The method also required large sample volumes and large quantities of extraction solvents which rendering the whole process unnecessarily expensive for laboratories with limited resources. Xiangjun *et al.*, 2014; developed an HPLC-MS-MS method for the simultaneous quantitation of hydrochlorothiazide and irbesartan in human plasma.

Nevertheless, the method required solid phase extraction cartridges, large sample volumes and complex sample preparation steps with large volumes of extraction solvents. As per the literature review, the other methods employed liquid-liquid extraction procedures using large sample volumes and extraction solvents with complex and laborious procedures.

In this study, we report the first simultaneous HPLC-ESI-MS/MS method that is rapid, simple, robust, sensitive and selective for simultaneous quantitation of HCTZ and NFP in a small volume (200 μ L) of human plasma. This method is suitable for processing and analysis of large number of HCTZ and NFP samples drawn during clinical pharmacokinetics or therapeutic drug monitoring studies.

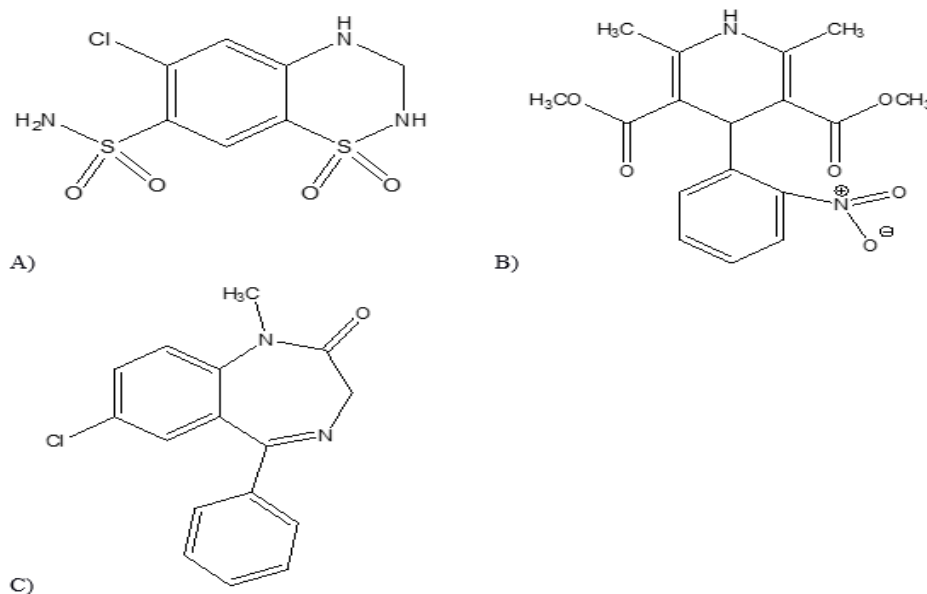


Figure 1. Chemical structures of hydrochlorothiazide (A), nifedipine (B) and diazepam, IS (C)

2. MATERIALS AND METHODS

2.1 Chemical and reagents

Hydrochlorothiazide (HCTZ; lot no. BCBL6365V, purity \geq 98.9%; MW=297.74 g/mol), nifedipine (NFP; lot no. MKBW8746V, purity \geq 98.0%, MW=346.33 g/mol) and diazepam (DZP, lot no. 105F0451, purity \geq 98.7%, MW=284.74 g/mol), acetonitrile and methanol (both LC-MS grade), ethyl acetate and dichloromethane (both HPLC grade), formic acid (85%; AnalaR[®] grade) and ammonium formate (AnalaR[®] grade) were purchased from Sigma Aldrich (St. Louis, MO, USA). Deionized water was prepared using a Smart2 Pure[™] water purification system (Thermo-scientific, Niederelbert, Germany). Blank human plasma with Li-heparin for the preparation of calibrators and quality controls was obtained from Kenya Medical Research Institute, Centre for Clinical Research (Nairobi, Kenya).

2.2 Instrumentation

The LC system consisted of an Agilent Technologies HPLC-ESI-MS/MS system (Santa Clara, CA, USA), composed of a 1260 μ Quaternary Pumps, 1260 Autosampler and 1260 Thermo-setting Column Compartment (TCC).

Chromatographic separation was performed on a Polaris 5 C18-A (150 mm x 3.0 mm I.D; 3.0 μ m particle size) analytical column from Agilent Technologies (Santa Clara, CA, USA) with a C18 guard cartridge (4 mm x 3.0 mm, 3.0 μ m) (Phenomenex, Torrance, CA, USA) maintained at 40 $^{\circ}$ C. The mobile phase consisted of (A) 5mM aqueous ammonium formate pH 6.0 and (B)

0.1% formic acid in methanol. A linear gradient elution was used to deliver the mobile phase, 60% solvent B at time 0 min, and 90% from 1.5 min, to 4.2 min, and back to 60% from 5.5 min to 10 min, (re-equilibration step). The flow rate was set at 300 $\mu\text{L}/\text{min}$, the autosampler was conditioned at 18 $^{\circ}\text{C}$ and injection volume of 20 μL was used to optimize the drug signals and for analysis.

Mass spectrometric detection of analytes was performed on a 6410 Triple Quadrupole Mass Spectrometer with an Electrospray Ionization (ESI) source from Agilent Technologies (Santa Clara, CA, USA) in both positive and negative ionization modes. Nitrogen was used as the nebulizing, desolvation and collision gas, the optimized ion source parameters were: ion spray voltage 4.0 kV, exit potential 7V, RF lens 0.5V.

Source temperature was 100 $^{\circ}\text{C}$ and desolvation temperature 350 $^{\circ}\text{C}$. High purity nitrogen from Genius NM32LA generator (Peak Scientific, Scotland, UK) was used as both sheath and auxiliary gas set at 20 l/min and 12 l/min, respectively.

Multiple reaction monitoring (MRM) was employed for the data acquisition, the analytical parameters optimized for the compounds were declustering potentials (DP) and collision energies (CE) (Table S1), and the scan dwell time was set at 500 ms for each channel. Data acquisition and analysis were accomplished with Mass Hunter software (version A.02.00; Agilent Technologies).

2.3 Extraction optimization

Liquid-liquid extraction was adopted for the extraction of HCTZ and NFP from plasma samples. Several extraction solvents including n-hexane-dichloromethane (3:2), diethyl ether-dichloromethane (7:3) and ethyl acetate-dichloromethane (8:2) were investigated. However, ethyl acetate-dichloromethane (8:2) that has been employed in this paper gave higher extraction recoveries for both HCTZ and NFP, could be evaporated easily and thus shortened the extraction time.

2.4 Preparation of calibration standards and quality control samples

The stock solutions of hydrochlorothiazide, nifedipine and diazepam (1mg/mL) were prepared by accurately weighing 2 mg of each substance and dissolving in 2 mL of acetonitrile in amber glass vials separately. Nifedipine solution was protected from light by wrapping with aluminium foil, the stock solutions were stored at - 20 $^{\circ}\text{C}$ and were stable for at least 3 months. Hydrochlorothiazide and nifedipine stock solutions were further diluted with 50% ACN to give working solutions of 20, 15, 10, 7, 4, 2, 1, 0.05 $\mu\text{g}/\text{mL}$ (HCTZ) and 4, 3, 2, 1.4, 0.8, 0.4, 0.2, 0.05 $\mu\text{g}/\text{mL}$ (NFP); all the working solutions were protected from light and stored at - 20 $^{\circ}\text{C}$ where they were stable for at least 3 weeks. The diazepam (IS) working solution used was 100ng/mL.

Calibration standard samples were freshly prepared by spiking 400 μL of blank plasma with 50 μL of HCTZ and 50 μL of NFP calibration solutions to yield a series of standards for calibration corresponding to 5, 100, 200, 400, 700, 1000, 1500, 2000ng/mL (HCTZ) and 5, 20, 40, 80, 140, 200, 300 and 400ng/mL (NFP). Quality control (QC) samples were prepared at low (15ng/mL), medium (800ng/mL), high (1600ng/mL) for HCTZ and at low (15ng/mL), medium (160ng/mL), and high (320ng/mL) for NFP. The QC samples were prepared in the same way as the plasma samples for calibration using appropriate working solutions with 400 μL of blank plasma.

2.5 Sample preparation

Blood samples were collected in Li- heparin tubes. After centrifugation at 3000 x g for 10 min, the separated plasma was collected and stored from light at -70 °C till the day of analysis. Before analysis, the plasma samples were allowed to thaw unassisted at room temperature. To a 200µL aliquot of plasma (blank, calibrator, quality control, or patient sample) in a 1.5mL polypropylene tube, 50µL of the internal standard (DZP; of a 100ng/mL solution in acetonitrile) was added and subjected to liquid-liquid extraction with 1ml of ethyl acetate-dichloromethane (8:2) as the extraction solvent. The tubes were vortex-mixed for 3 minutes followed by centrifugation on a Thermo Fisher Scientific SL 40R centrifuge (4000 x g; 10 min, 4 °C). The supernatant organic layer (1mL) was quantitatively transferred to another clean 1.5mL polypropylene tube and evaporated to dryness under a stream of nitrogen on a Grant JB Series water bath (Grant Instruments, Cambridge, UK) at 40 °C.

The residue was reconstituted with 100µL of mobile phase (A-B, 20:80) and vortex-mixed for 3 minutes, transferred into autosampler vial and submitted for analysis with a volume of 20µL being injected onto the column.

2.6 Method validation

The method was validated for selectivity, linearity, precision, accuracy, extraction recovery, matrix effect, carry over and stability according to the US Food and Drug Administration Guidance for Industry Bioanalytical Method Validation (van Amsterdam et al., 2013).

2.6.1 Selectivity

Selectivity of the method was assessed and assured by analysis of six independent blank plasma samples from different sources, each blank sample was tested for interference using the proposed extraction procedure and chromatographic/mass spectrometric conditions and compared with those obtained with an aqueous solution of the analytes at concentrations near to the lower limit of quantification (LLOQ).

2.6.2 Linearity and LLOQ

Linearity of the calibration curves were assessed by assaying standard plasma samples at eight concentrations in the range of 5–2000 ng/mL for HCTZ and 5 – 400 ng/mL for NFP on three consecutive days. The curves were constructed by plotting the peak-area ratio of each analyte to that of the IS versus the nominal concentration of the drugs. The curves were evaluated and fitted by weighted (1/x) linear regression. The LLOQ was defined as the lowest concentration on the calibration curve, at which an acceptable accuracy (relative error, RE) within $\pm 20\%$ and a precision (relative standard deviation, RSD) below 15% can be obtained by means of analyses of at six replicates from a homogenous sample.

2.6.3 Accuracy and precision

The intra-day accuracy and precision were evaluated by analyzing six replicates of QC samples (15, 800, 1600 ng/mL) for hydrochlorothiazide and (15, 160, 320 ng/mL) for nifedipine on the same day. Inter-day accuracy and precision was determined by analyzing six replicates of freshly prepared QC samples on three consecutive days.

Each validation run consisted of one set of calibration standards and six replicates of QC samples at three concentration levels.

2.6.4 Extraction recovery and matrix effect

The extraction recovery of the method was determined by analyzing six replicates of plasma samples at three QC concentration levels of 15, 800, 1600 ng/mL (HCTZ) and 15, 160, 320 ng/mL (NFP) corresponding to low, medium and high QCs. The recovery was calculated by comparing the peak areas obtained from extracted spiked samples (A) with those of samples spiked post-extraction (B) at corresponding concentrations. To evaluate the matrix effect, HCTZ and NFP at two concentration levels (low and high QCs) were added to the extract of 200 μ L of blank plasma, dried and reconstituted with 100 μ L of mobile phase A-B (20:80, v/v). Absolute matrix effect (expressed as matrix factors) was assessed by comparing the mean area responses of post-extraction fortified samples (B) to those of neat samples (C) prepared in elution solution. Ion suppression/enhancement effects on the MRM LC-MS/MS sensitivity were evaluated by post column analyte infusion experiment. A standard solution containing IS, HCTZ and NFP (at ULOQ level) were infused post column into the mobile phase at 5 μ L/min employing infusion pump. Aliquots of 5 μ L of extracted control blank plasma sample were then injected into the column and chromatograms were acquired for the analytes and IS.

$$MF = \frac{B}{C} \quad (i)$$

The extraction recovery and matrix effect of IS at a single concentration of 50 ng/mL were also evaluated using the same procedure.

2.6.5 Carry-over

A processed blank sample was injected after a high concentration calibration standard at the upper limit of quantification (ULOQ) and the resulting response evaluated in respect of any significant drag quantity transferred from the high concentration calibration standard.

2.6.6 Stability

The stability of HCTZ and NFP in human plasma was assessed by analyzing six replicates of low and high QC samples under different temperature and time conditions. The freeze-thaw stability was performed by subjecting QC plasma samples to three freeze (-20°C)–thaw cycles at ambient temperature (24°C). QC samples were stored frozen at -20°C for 60 days and analyzed after allowing to thaw unassisted at ambient temperature to determine the long-term stability. Short-term stability was assessed by keeping QC samples at ambient temperatures for 8 h before processing and analyses. The post-preparation stability was studied by analyzing the processed QC samples kept in the autosampler at 4°C for 15 h. Sub-stock solution stability was evaluated for HCTZ, NFP and DZP, by comparing the response generated from the same solution at preparation and after being stored at -20°C for a period of 28 days. The analytes were considered stable when the concentrations found were within $\pm 15\%$ of the initial concentration.

$$ST\% = \frac{c_t}{c_0} \times 100\% \quad (ii)$$

Where c_0 is the initial concentration, determined without introducing any extra pauses in the analysis process; c_t is the concentration obtained after the storage period with time t .

2.7 Pharmacokinetics study

The validated method was used to determine the plasma concentrations of hydrochlorothiazide and nifedipine from patients being treated for hypertension. The pharmacokinetic study was approved by the Strathmore University Ethics Committee and carried out at Mbagathi County hospital facility in Nairobi, Kenya after a signed informed consent was received from the study participants. Blood samples were collected as the patients came for their routine medical care; the prescription, drug type and dosage strengths were determined by the doctors in charge and varied between patients. The plasma was immediately separated by centrifugation at 2000 x g for 10 min and frozen at $-80\text{ }^{\circ}\text{C}$ until analysis. The study protocol was approved by the Ethics Committee of Kenya. The two drugs hydrochlorothiazide and Nifedipine were the most commonly used by patients being treated of hypertension at Mbagathi County Hospital.

3. RESULTS AND DISCUSSION

3.1 Optimization of mass spectrometry

The LC–MS/MS operation parameters were carefully optimized for the simultaneous determination of hydrochlorothiazide and nifedipine. The mass spectrometer was tuned in both positive and negative ionization modes with ESI for optimum response of both analytes. It was found that the HCTZ signal intensity of negative ion was higher than that of positive ion, whereas NFP and the IS generated higher signal intensities in the positive ion mode. In the precursor ion full-scan spectra, the most abundant ions were protonated molecules $[\text{M}+\text{H}]^+$ at m/z 347.2 and 285.0 for NFP and IS, respectively; and deprotonated molecules $[\text{M}-\text{H}]^-$ at m/z 296.1 for HCTZ. Parameters such as desolvation temperature, ESI source temperature, capillary and cone voltage, flow rate of desolvation gas and cone gas were optimized to obtain the highest intensity of the molecular ions of HCTZ and NFP. The full scan product ions mass spectra (Figure. 2) showed high abundance fragment ions at m/z 205.2 and m/z 315.1 for HCTZ and NFP respectively.

The declustering potential and collision voltage of collision-induced dissociation (CID) were optimized for maximum response of the fragmentation ions for the analytes. The precursor \rightarrow product ion transitions of m/z 347.2 \rightarrow 315.1 for NFP, m/z 296.1 \rightarrow 205.2 for HCTZ and m/z 193.1 \rightarrow 285.0 for IS were chosen for MRM. No cross-talk was observed between the MRMs of the analytes.

In studies by Dipanjan *et al.*, 2017, Wang *et al.*, 2011 and Xiangjun *et al.*, 2014, the full scan product ions mass spectra for NFP and HCTZ have been shown, however, we report a more informative product ions mass spectra with the fragmentation patterns of the molecular ions.

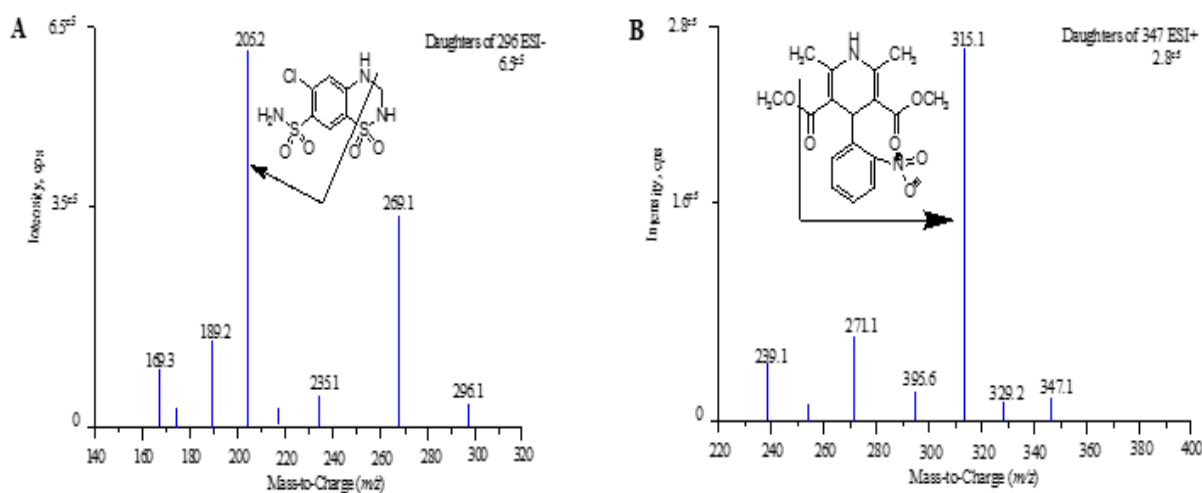


Figure 2. Full scan product ions mass spectra of $[\text{M}-\text{H}]^-$ of hydrochlorothiazide (A) and $[\text{M}+\text{H}]^+$ of nifedipine (B). The most intense ions were monitored and used for quantitation.

3.2 Optimization of chromatography

During the method development, several chromatographic conditions were optimized such as the mobile phase composition, mobile phase pH and flow rate. Various ratios (65:35, 70:30, 60:40 v/v) of acetonitrile and 10mM ammonium formate were tested as starting eluent for chromatographic separation. This composition however, did not produce desirable intensities of HCTZ since it required a hydroxyl (OH) group to facilitate deprotonation. In view of this, acetonitrile was replaced with methanol.

The ionization of NFP and IS was increased by adding additives to the mobile phase. Therefore, formic acid and ammonium formate were attempted to improve the analyte response. When formic acid was added to the mobile phase, the peak shapes and sensitivity of the protonated molecular ions (NFP and IS) improved significantly. Chromatographic analysis was tested on three different reversed phase columns namely, Eclipse plus C18 (50 mm×2.1 mm, 1.8 μm), Zorbax Polaris 3 C18 (100 mm×3 mm, 3.0 μm) and Zorbax Polaris 5 C18 (150 mm×3.0 mm, 3 μm).

For mobile phase selection, different combinations of acetonitrile/methanol and acidic buffers (ammonium formate/ammonium acetate) in the pH range of 3.0–6.5 were tested to have adequate retention, sufficient response and sharp peak shapes. Acetonitrile as organic modifier provided higher sensitivity than methanol for NFP, a compromise to use methanol was to enable simultaneous quantification of both HCTZ and NFP. Similarly, the signal obtained employing ammonium formate buffer was higher than ammonium acetate buffer for both the drugs. Although baseline separation ($R_s \geq 1.78$) was achieved on all three columns, Zorbax Polaris 5 C18 provided the desired sensitivity, adequate retention and good peak shape for both the analytes using methanol and 5.0 mM ammonium formate (pH 6.0, adjusted with ammonium solution). Therefore, in the present method, buffer pH adjusted to 6.0 and the LC gradient chosen ensured sharp chromatographic peaks with the best possible baseline-resolved separations of HCTZ, NFP and DZP (IS) within 8 minutes and a total runtime of 10 minutes.

3.3 Selection of extraction method

Several extraction methods were tried, since HCTZ, NFP and DZP (IS) significantly differs in drug-plasma binding and physicochemical properties, it was difficult to optimize extraction procedure for all the analytes and IS from plasma. Several approaches were tried based on the previously reported methods such as protein precipitation (PPT), solid phase extraction (SPE) and liquid-liquid extraction (LLE). Finally LLE procedure was chosen, being economical compared to the SPE used in previous publications (Xiangjun *et al.*, 2014; Ramkumar *et al.*, 2014). The extraction procedure was also optimized to allow the use of 1.5mL polypropylene tubes thus eliminating drug loss resulting from use of larger tubes and use of large volumes of extraction solvents (Wang *et al.*, 2011). Whereas several extraction solvents including n-hexane-dichloromethane (3:2), diethyl ether-dichloromethane (7:3) and ethyl acetate-dichloromethane (8:2) were investigated, ethyl acetate-dichloromethane (8:2) that has been employed in this paper gave higher extraction recoveries for all the analytes and could be evaporated easily and thus shortened the extraction time.

3.4 Method validation

3.4.1 Selectivity

All the lots of blank plasma used for selectivity studies met the acceptance criteria selectivity. Figure 3 (A- C) shows the typical chromatograms of extracted double blank, blank plasma (containing IS only) and spiked plasma sample with the analytes at LLOQ and ULOQ level. These

results supported the high selectivity and specificity of this method as there were no interfering peaks from endogenous compounds observed at the retention times of the analytes and the IS.

3.4.2 Linearity and limit of quantification

Calibration curves were constructed by plotting eight calibration standards of HCTZ and NFP. The curves for analytes spiked in plasma were found to be linear over the concentration ranges of 5-2000 ng/mL (HCTZ) and 5-400 ng/mL (NFP). A weighted ($1/x$) linear regression model was used by determining the best fit of the peak-area ratios (peak area of analyte/ peak area of IS) vs analyte concentration and conformed to $y = mx + c$ (where y - peak area ratio; x - concentration; m - slope of the curve). The choice of this regression model was based on all available data from the validation phase, in light of this the method proved to be reliable in terms of accuracy and reproducibility over the entire calibration range. Typical regression equations for the calibration curves were $y = 2.2409 \times 10^{-2} x + 0.048436$ ($r^2 = 0.9967$) for NFP and $y = 1.91419 \times 10^{-5} x + 3.420299 \times 10^{-5}$ ($r^2 = 0.9958$) for HCTZ (Table 1). The coefficients of variation for the slopes of six calibration curves were 9.4% (HCTZ) and 7.2% (NFP). The LLOQs were determined by the needs of the method and defined as the lowest standards on the calibration curve with identifiable, discrete, and reproducible with a precision $\leq 20\%$ and accuracy within 85%-115% (Table 2). LLOQ was 5 ng/mL for both the analytes and was found adequate for the clinical PK studies and drug level monitoring following oral administration of therapeutic doses of HCTZ and NFP. The limits of detection (LODs) were determined as the lowest concentration of the analyte at which the signal to noise (S/N) ratio exceeded 3:1 (van Amsterdam *et al.*, 2013), these were 2 ng/mL and 0.5 ng/mL for HCTZ and NFP respectively.

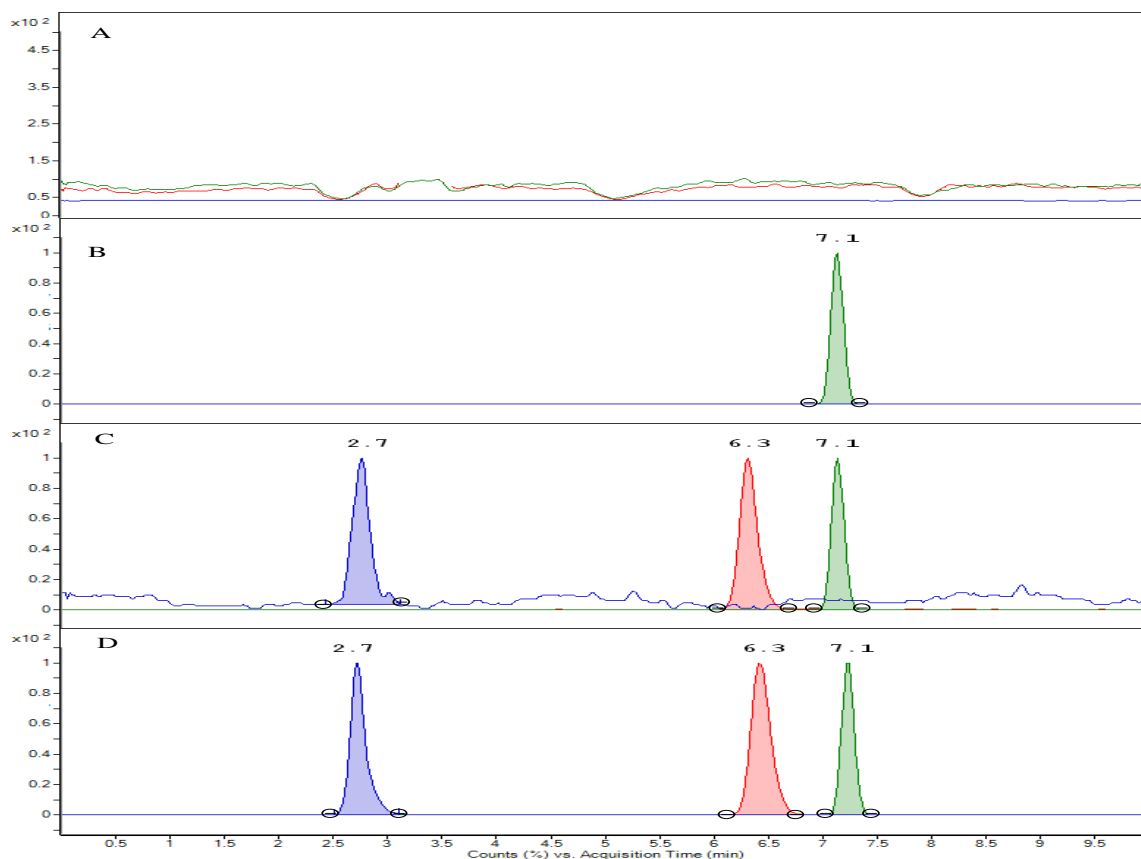


Figure 3. Representative overlaid chromatograms of extracted plasma samples of a double blank (A), a blank with IS only (B), LLOQ (C) and ULOQ (C) with HCTZ (RT 2.7 min), NFP (RT 6.3 min) and DZP (RT 7.1 min).

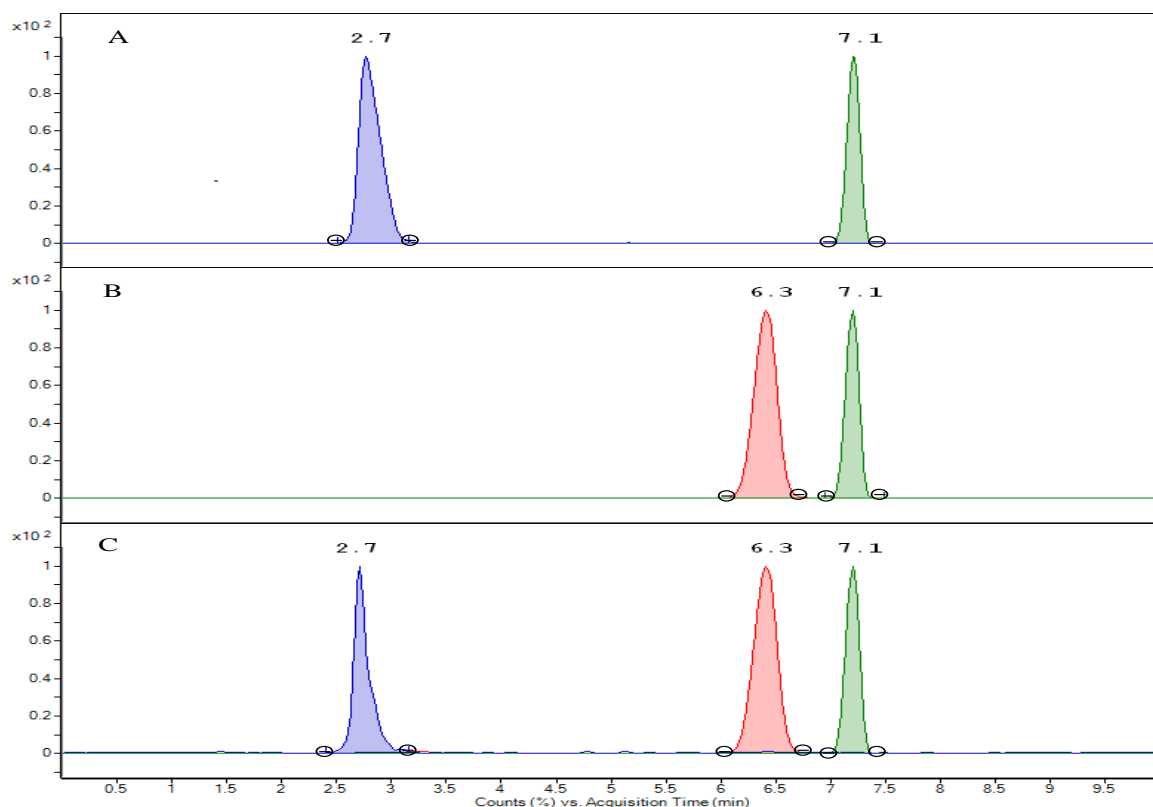


Figure 4. Representative overlaid chromatograms of extracted patient samples containing HCTZ only (A), NFP only (B) and both HCTZ and NFP (C) with HCTZ (RT 2.7 min), NFP (RT 6.3 min) and DZP (RT 7.1 min).

Analyte	Standard concentration (ng/ mL)	Mean estimated concentration (ng/ mL)	SD	Precision (CV %)	Nominal (%)	Slope	Intercept	r ²
HCTZ	5	4.8	0.3	6.1	95.4	1.914E-05	3.420E-05	0.9958
	100	100.7	4.5	4.5	100.7			
	200	207.3	18.2	8.8	103.7			
	400	429.0	27.1	6.3	107.2			
	700	676.2	28.6	4.2	96.6			
	1000	1004.8	56.0	5.6	100.5			
	1500	1466.1	112.7	7.7	97.7			
	2000	2018.5	87.1	4.3	100.9			
NFP	5	5.4	0.5	8.9	108.4	2.240E-02	4.843E-02	0.9958
	20	19.2	1.4	7.2	95.8			
	40	38.6	3.3	8.5	96.5			
	80	79.1	7.1	9.0	98.9			
	140	133.3	8.2	6.2	95.2			
	200	197.1	7.7	3.9	98.5			
	300	298.7	10.2	3.4	99.6			
	400	411.3	16.7	4.1	102.8			

Table 1. Back-calculated concentrations of calibration standards for hydrochlorothiazide (HCTZ) and nifedipine (NFP) in plasma (n=3).

Analyte	Level	Added concentration (ng/ mL)	Intra-day (n=6)			Inter-day (n=18)		
			Mean estimated concentration (ng/ mL)	Precision (CV %)	Nominal (%)	Mean estimated concentration (ng/ mL)	Precision (CV %)	Nominal (%)
HCTZ	LLOQ	5	4.6	5.2	91.9	4.8	6.0	95.3
	LQC	15	14.9	5.7	99.1	15.4	3.0	102.6
	MQC	800	830.7	3.2	103.8	794.6	5.4	99.3
	HQC	1600	1568.4	1.8	98.0	1603.9	3.0	100.2
NFP	LLOQ	5	5.2	4.0	104.8	5.4	8.9	108.4
	LQC	15	14.1	5.7	93.9	14.3	3.2	95.6
	MQC	160	154.3	2.8	96.5	157.6	6.6	98.5
	HQC	320	321.7	5.9	100.5	318.6	1.1	99.6

Table 2. Intra-assay and inter-assay accuracy and precision of hydrochlorothiazide (HCTZ) and nifedipine (NFP) in plasma at LLOQ, LOQ, MOQ and HQQ.

3.4.3 Accuracy and precision

To evaluate the inter-assay precision and accuracy, six replicates of quality control (LLOQ, LQC, MQC and HQC) plasma samples were analyzed together with one independent calibration standard curve, this was done in three consecutive days; while intra-assay precision and accuracy were evaluated by analysis of quality control plasma samples in replicate of six in the same day. Inter-assay and intra-assay precision were expressed as coefficient of variation (CV %). The accuracy was expressed as the percent ratio between the experimental concentrations and the nominal concentration for each sample. Assay accuracy and precision data shown in Table 2.

3.4.4 Extraction recovery and matrix effect

The mean extraction recoveries of analytes from human plasma were $98.6 \pm 0.6\%$ (HCTZ), $97.4 \pm 3.1\%$ (NFP) and $89.8 \pm 2.4\%$ for the IS (Table 3). The recoveries reported in this method were significantly higher than those in previously reported publications (Ramakrishna N. *et al.*, 2005; Shah H. *et al.*, 2009; Olga S. *et al.*, 2014; Xiangjun *et al.*, 2014; Ramkumar *et al.*, 2015; Jaivik V. *et al.*, 2017) for HCTZ and (Wang *et al.*, 2011; Hisham S. *et al.*, 2000; Yunzhen G. *et al.*, 2007; Essam E. *et al.*, 2014) for NFP. This method yielded higher recoveries with better reproducibility due to the use of 1.5mL polypropylene tubes in sample processing compared to other reported methods that used larger tubes (15mL) hence loss of drugs along the walls of the tubes. Protein precipitation method of sample preparation is known to be prone to matrix effect, furthermore recovery of analytes with strong interactions with plasma proteins is usually a challenge that cannot be ignored.

NFP (pKa 2.60) is acidic while HCTZ (pKa 7.9 and 9.2) is a basic drug and their extraction from human plasma has been reported using all three conventional extraction techniques, namely, protein precipitation (PPT) (Essam E. *et al.*, 2014; Song M. *et al.*, 2007), liquid-liquid extraction (LLE) (Yunzhen G. *et al.*, 2007; Ramkumar *et al.*, 2015) and SPE (Shah J. 2017). As reported by Shah J. 2017, the mean extraction recovery for HCTZ obtained using SPE was 98.7%. However, Song M. *et al.*, 2007 reported quantitative recoveries in the range of 82.2%–98.9% for HCTZ using PP with acetonitrile. Nevertheless, based on our work on extraction recovery optimization, LLE was used in the present work to obtain clear extracts with less interference and thus obtaining higher sensitivity. Correct choice of solvent

for sample reconstitution was essential to obtain sharp chromatographic peaks with the best possible base-line resolution and was successfully achieved using 5mM ammonium formate and 0.1% formic acid in methanol (20:80 v/v).

Highly precise and quantitative recovery was obtained for NFP (94.3%–100.4%) and HCTZ (96.6%–99.0%) under the established extraction conditions using 200µL plasma sample. There was minimal interference from the matrix effect as evident from the IS-normalized matrix factors, which ranged from 0.98 to 1.06 for both the drugs (Table 3).

3.4.5 Carry over

The carry over in the blank sample following the high concentration standard was less than 20% of the response of the lower limit of quantification (LLOQ) of both HCTZ and NFP, and less than 5% the IS response. The effect of the carry over on the method performance was therefore insignificant.

3.4.6 Stability

The stability of HCTZ and NFP were evaluated at length both in stock solutions and in plasma samples under varied storage conditions. Sub-stock solutions stability studies undertaken assured analyte stability for a minimum of 28 days at refrigerated temperature (-20 °C), to ensure data integrity however, fresh stock solutions were prepared for all the analytes within the 28 days of use.

Level (ng/mL)	Mean estimated Concentration (ng/mL)			Recovery (A/B %)		Matrix factor (B/C)		
	Plasma spike (A)	Post ex- traction spike (B)	Neat spike (C)	Analyte	IS	Analyte	IS	IS-normalized (Analyte/IS)
HCTZ								
15	14.9	15.4	15.5	96.6	91.8	0.98	0.96	1.02
800	780.6	788.9	795.6	99.0	87.8	0.99	1.01	0.98
1600	1579.9	1598.5	1587.2	98.8	89.7	1.01	0.98	1.03
NFP								
15	14.9	15.8	15.28	94.3	89.6	1.03	0.98	1.06
160	154.5	158.6	160.12	97.4	93.2	0.99	0.95	1.04
320	320.6	318.7	321.3	100.4	86.9	0.99	0.98	1.01

Table 3. Extraction recovery and matrix factor for hydrochlorothiazide (HCTZ) and nifedipine (NFP); n=6.

Stability parameters	Spiked Conc. (ng/ mL)	HCTZ		NFP	
		15	1600	15	320
Bench-top stability in matrix (room temperature, 8 h)	Mean of stability samples	14.3	1533.0	14.4	307.9
	CV %	4.9	4.1	4.5	3.1
	*ST %	95.8	95.8	95.9	96.2
Freeze-thaw stability (3 freeze-thaw cycles at -20 °C)	Mean of stability samples	14.8	1540.9	13.9	307.2
	CV %	4.5	2.6	3.2	3.1
	*ST %	99.0	96.3	92.7	96.0
Auto-sampler (15 h at 4 °C)	Mean of stability samples	14.3	1635.3	14.5	296.2
	CV %	7.1	5.2	2.7	7.2
	*ST %	95.1	102.2	96.8	92.6
Long-term (60 days at -20 °C)	Mean of stability samples	14.3	1500.8	14.1	293.4
	CV %	3.0	4.1	3.2	4.3
	*ST %	95.2	93.8	94.2	91.7
Sub-stock solution (28 days at -20 °C)	stability	Norminal Conc. (µg/ mL)		100	50
		Mean of stability samples		98.8	48.6
		CV %		2.8	3.1
		*ST %		98.8	97.1

Table 4. Stability of hydrochlorothiazide (HCTZ) and nifedipine (NFP) in plasma under different conditions (n=6); *ST% = [Mean concentration of stability sample/ Mean concentration of comparison samples] x100

Spiked plasma samples were subjected to three freeze-thaw cycles and the analytes concentrations assessed after the third cycle. The freeze-thaw cycles were carried out in a -20 °C freezer. Freeze and thaw stability data presented in Table 4 showed that all the analytes were stable (92.7% - 99%) with analytically acceptable precision (2.6% - 4.5%). Assessment of bench-top stability was achieved through low and high quality control samples kept at room temperature for a minimum of eight hours, processed and analyzed. Jaivik V. *et al.*, 2017 reported stabilities of hydrochlorothiazide over a longer duration than in this method. Our choice for the 8 h period was to report an analytically relevant duration under which the two drugs can be analyzed considering the photo instability of nifedipine.

The results indicated that the drugs were stable through the 8h period and therefore the sample processing procedure outlined within this method is applicable in processing large number of samples without the risk of sample degradation due to room temperature exposure.

The results of post processing stability (auto-sampler stability) indicated that all the drugs were stable after 15 h in the autosampler and the integrity of data obtained after re-assay within this time post-processing would not be questionable. Jaivik V. *et al.*, 2017 reported the autosampler stability of HCTZ for 36 h, however the long-term stability data reported in his study (176 days, -20 °C) showed a concentration change in stability samples ranging from -1.83% to -3.59%.

The long term stability data reported in this study (for 60 days) show that all the analytes were stable (91.7% - 95.2%) within the period investigated. Since the stability at -20 °C was acceptable, there was no need to evaluate the stability at -80 °C as our aim was to report a method that is applicable to resource limited laboratories.

3.5 Method application to real patient samples

The validated method was successfully applied to a pharmacokinetic study in volunteers under hypertension management care. The sensitivity and specificity of the method proved adequate for reliably characterizing the pharmacokinetics of hydrochlorothiazide and nifedipine.

The patient samples were successfully analyzed using this method and no interference of endogenous compounds resulting from altered plasma protein compositions was encountered.

4. CONCLUSION

The validated HPLC–ESI–MS/MS method is the first report for the simultaneous quantitation of hydrochlorothiazide and nifedipine from only 200µL human plasma. It provided simple and rapid analyses, as well as sensitive and reliable results. Thus, this method is suitable for routine high-throughput analyses and may be successfully applied to pharmacokinetic, therapeutic drug monitoring and bioequivalence of multiple doses evaluated in the present work in human subjects.

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Conflict of Interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the

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