Simultaneous determination of bisoprolol and hydrochlorothiazide in human plasma by HPLC coupled with tandem mass spectrometry

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ABSTRACT

A sensitive, specific and selective method has been developed for the simultaneous determination of bisoprolol and hydrochlorothiazide in human plasma. The method employed a state of the art LC–MS/MS operated in the positive and negative ionization switching modes. A simple sample preparation step involving protein precipitation with acetonitrile has been optimized; the analytes and the internal standard moxifloxacin were separated on a Purosphere® STAR C_8 column (125 mm × 4 mm, 5 µm). The mobile phase was an ammonium acetate solution (1 mM) with formic acid (0.2%): methanol and acetonitrile (65:17.5:17.5, v/v/v (%)), the flow rate was set at 0.65 mL/min. Bisoprolol and hydrochlorothiazide were ionized using ESI source prior to detection by Multiple Reaction Monitoring (MRM) mode while monitoring at the following transitions: positive m/z 326 → 116 for bisoprolol, negative m/z 296 → 269 and m/z 296 → 205 for hydrochlorothiazide. Linearity was demonstrated over the concentration range 0.10–30.0 (ng/mL) for bisoprolol and 1.00–80.00 ng/mL for hydrochlorothiazide. The limits of detection were 0.100 (ng/mL) for bisoprolol and 1.00 (ng/mL) for hydrochlorothiazide. The validated method was successfully applied to a pharmacokinetic study of 5 mg bisoprolol fumarate with 12.5 mg hydrochlorothiazide tablet in healthy volunteers.

1. Introduction

Bisoprolol (BISO) and hydrochlorothiazide (HCTZ) have been recently suggested as a combination therapy for the treatment of hypertension and chronic heart failure. Reports indicate that low doses of BISO and HCTZ demonstrate synergistic and antihypertensive effects and provide optimal therapeutic benefits in rats [1,2].

HCTZ, 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide (Fig. 1A), is one of the oldest thiazide diuretics, often described in combination with other drugs such as β-blockers, ACE inhibitors, or angiotensin II receptor blockers [3]. BISO, 1-[4-[[2-(1-methylethoxy)ethoxy]methyl]phenoxy]-3-[(1-methylethyl) amino2-Propanol (Fig. 1B), is a highly selective β_1-receptor blocking agent used for the treatment of hypertension and angina pectoris [4].

As a result of the recent developments and the suggested BISO-HCTZ combination therapy, simultaneous determination of BISO and HCTZ in human plasma should facilitate fast and more efficient evaluation of pharmacokinetic and bioavailability data, in addition to bioequivalence studies.

Several chromatographic methods have been reported for the analysis of either BISO or HCTZ alone in human plasma; such methods have included HPLC coupled with UV [5-7] or fluorescence [8], diode array [9], LC–MS [10–12] or with tandem LC–MS/MS detection [13–19].

Till this day, the simultaneous determination of BISO and HCTZ has not been reported. This may be due to the difficulty in extracting and detecting both active ingredients simultaneously in the plasma matrix, in addition the following should be addressed (1) both analytes have different structural and spectral properties [20], (2) both have different polarities and solubilities (Table 1) [21–25], (3) spectrophotometric techniques with limited sensitivities cannot simultaneously measure the required limits of the quantitation anticipated for both HCTZ and BISO (4) for the LC–MS/MS, a positive ionization mode is required to detect BISO, whereas a negative ionization mode is required to detect HCTZ, consequently a high HPLC resolution is mandatory.

The present work reports a sensitive and selective tandem LC–MS/MS method for the simultaneous determination of BISO and

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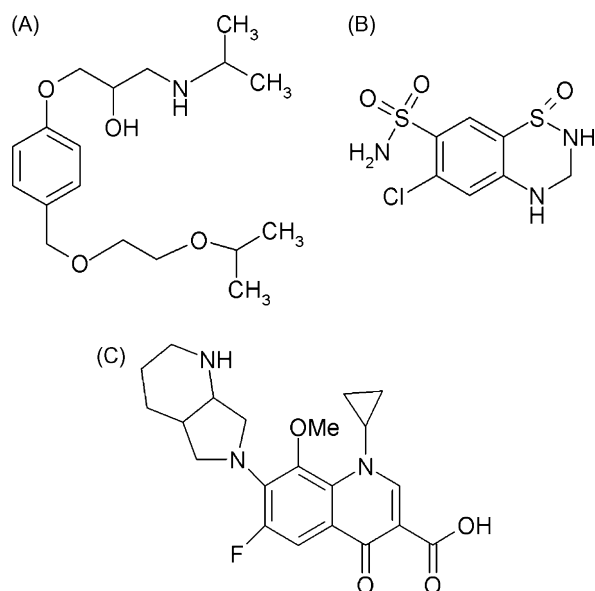   [2] Tel. +962 6 5355000x23382; fax: +962 6 4622446.

Fig. 1. Structures of (A) bisoprolol, (B) hydrochlorothiazide and (C) moxifloxacin.

HCTZ in human plasma after dosing of 5 mg BISO combined with 12.5 mg HCTZ tablet to healthy volunteers in fasting state.

2. Experimental

2.1. Chemicals and reagents

Working standards of bisoprolol fumarate (99.5%), hydrochlorothiazide (101.4%) and moxifloxacin hydrochloride (Fig. 1C) (99.5%) used as an internal standard (IS) were kindly provided by Jamjoom pharmaceuticals (Jeddah, Saudi Arabia). HPLC gradient grade acetonitrile and methanol, in addition to analytical grade formic acid and ammonium acetate were purchased from Merck (Darmstadt, Germany). All reagents were used without further purification. Blank human plasma was obtained

from the blood bank (Amman, Jordan) and stored at −30 °C prior to use. Water was purified using a Sartorius arium 611 UV water purification system which provides ASTM Type I water, with low total organic content (TOC < 1 ppb).

2.2. Instrumentation

The LC–MS/MS system consisted of a high performance liquid chromatograph (Agilent 1200 Series, Agilent Technologies, Waldbronn Germany) coupled with an Applied Biosystems Sciex a triple quadrupole mass spectrometer (API 5000,MDS Sciex, Ontario, Canada), equipped with electrospray ionization (ESI). Data acquisition and processing were controlled by Applied Biosystems/MDS SCIEX Analyst software (Version 1.4.2).

2.3. LC–MS/MS chromatographic conditions

Chromatographic separation was performed using a Purosphere® STAR C$_8$ column (125 × 4 (mm), 5 μm, Merck, Germany) thermostated at 50 °C. The mobile phase was an ammonium acetate solution (1 mM) with formic acid (0.2%): methanol and acetonitrile (65:17.5:17.5, v/v/v (%)). The separation was performed under isocratic conditions with a constant flow rate of 0.65 (mL/min), the injection volume was 10 μL.

LC–MS/MS experimental conditions utilized the multiple reaction monitoring (MRM), detection of bisoprolol and internal standard (moxifloxacin) was performed in the positive ESI mode for their respective [M+H]$^+$ ions, whereas detection of HCTZ was performed in the negative ESI mode for its [M−H]$^−$ ion. Instrument settings of the MS/MS are summarized in Table 2.

2.4. Standard solutions and calibrators

Stock standard solutions of BISO and HCTZ (400 (ng/mL)) each in acetonitrile) were freshly prepared A series of working standard solutions were diluted in acetonitrile to produce eight standard solutions ranging 10–3000 (ng/mL) for BISO and 100–8000 (ng/mL) for HCTZ, respectively. Matrix based calibrators were prepared by spiking 100 μL of each standard solution to a final volume of

Table 1
Relevant physiochemical properties of bisoprolol and hydrochlorothiazide.

Compound	pKa	LogP	Solubility
Bisoprolol	9.5	2.2	Readily soluble in water (2.24 mg/mL), methanol, ethanol and chloroform
Hydrochlorothiazide	8.6, 9.9	−0.5	Slightly soluble in water (0.7 mg/mL), soluble in aqueous NaOH and soluble in organic bases like n-butyl amine

Table 2
Experimental settings for the Tandem mass-spectrometer for the analysis of bisoprolol, hydrochlorothiazide and moxifloxacin (IS).

Parameter	Value		
Source temperature, °C	500		
Nebulizer gas, psi	60.0		
TurboIon gas, psi	60.0		
Curtain gas, psi	25.0		
Collision gas, psi	12.0		
	Bisoprolol	**Hydrochlorothiazide**	**Moxifloxacin**
Ion spray voltage, V	5500	−4500	5500
Declustering potential (DP), V	136.	−140	81
Mode of analysis	Positive	Negative	Positive
MRM transition (Collision energy, V)	326 → 116 (27.0)	296 → 269 (−26.0)	402 → 358 (29.0)
		296 → 205 (−32.0)	402 → 261 (35.0)
			402 → 231 (43.0)
			402 → 219 (59.0)
Dwell time per transition, ms	200	200	150

10.00 mL plasma. Quality control samples were prepared at 0.30, 15.00, and 24.00 (ng/mL) for BISO and 3.00, 40.00 and 64.00 (ng/mL) for HCTZ. A working standard solution containing 9.0 (ng/mL) of the IS was prepared in acetonitrile. Plasma solutions were stored in subdued light at −86 °C until assay.

2.5. Sample preparation

To an aliquot of 250 μL plasma in an Eppendorf tube, a 1.0 mL volume acetonitrile containing the IS (9.0 ng/mL) was added. The mixture was shaken (10 min) and centrifuged (4000 rpm for 5 min),
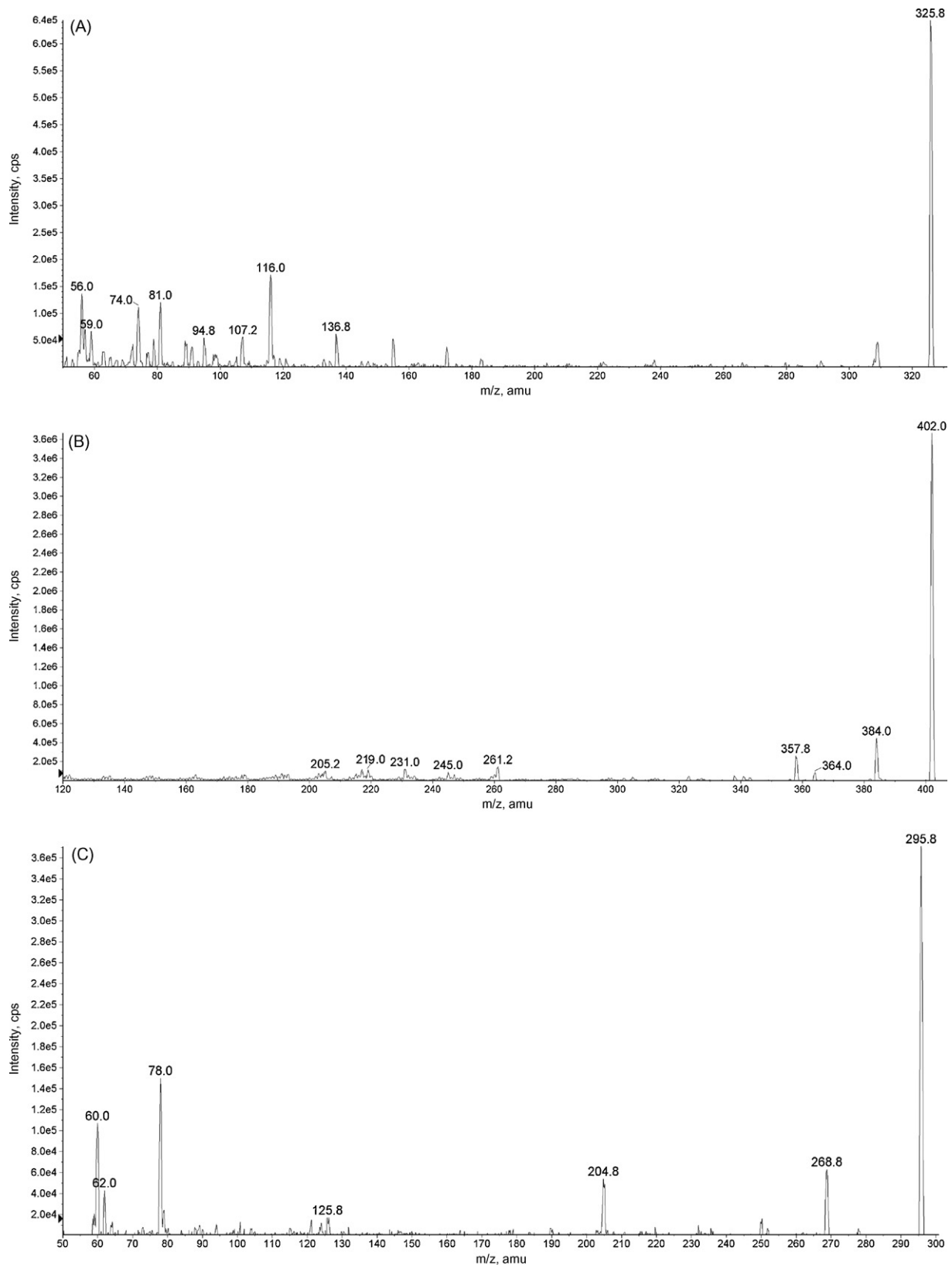

Fig. 2. A full-scan product ion mass spectra of 10.0 (ng/mL) in acetonitrile of [M+H]⁺ ions of (A) bisoprolol (B) moxifloxacin (IS), and [M-H]⁻ ion of (C) hydrochlorothiazide.

0.90 mL aliquot of the supernatant was transferred to a 10 mL glass tube. The mixture then evaporated to dryness, under a stream of nitrogen in a block heater (50 °C). The residue was reconstituted with 500 μL of the mobile phase; the mixture was vortex (30 s) and a 10 μL aliquot of the solution was injected into the LC–MS/MS system.

2.6. Method validation

Validation of the developed method was performed as outlined in the guidance of industry for bioanalytical method validation [26], selectivity, accuracy, precision, limit of quantitation, linearity, recovery and stability were investigated during the pre-study validation phase.

3. Results and discussion

3.1. Mass spectrometry

An LC–MS/MS system has been employed for the simultaneous determination of BISO and HCTZ in human plasma. The triple quadruple API 5000 offered high sensitivity and selectivity. Table 2 summarizes the optimum experimental conditions. Analytical signals of BISO and moxifloxacin (IS) were optimized in the positive mode, peak area of BISO was detected by the protonated molecule at m/z 326; the most abundant product ion at m/z 116. The peak areas of the moxifloxacin (IS) were monitored for its product ions at 358 and 261, the combined areas were utilized for calculating the area ratio for BISO determination. In order to minimize instrumental errors, an area ratio of about 1 was recommended for the middle concentration of the calibration graph. This was only achieved by combining areas of the two peaks corresponding to the IS. The above was also observed for evaluating HCTZ, which again utilized the combined peak areas of the HCTZ fragment ions at 269 and 205. Furthermore, the combined peak areas of the moxifloxacin (IS) at 231 and 219 were utilized to calculate area ratios as analytical signals for constructing calibration graph of HCTZ to enhance sensetivity. The mass spectra and proposed mechanism for the fragmentation processes are listed in Figs. 2 and 3, respectively.

3.2. Chromatography

Using a C_8 column (125 mm × 4 mm, 5 μm) the chromatographic conditions were optimized to achieve high resolution,
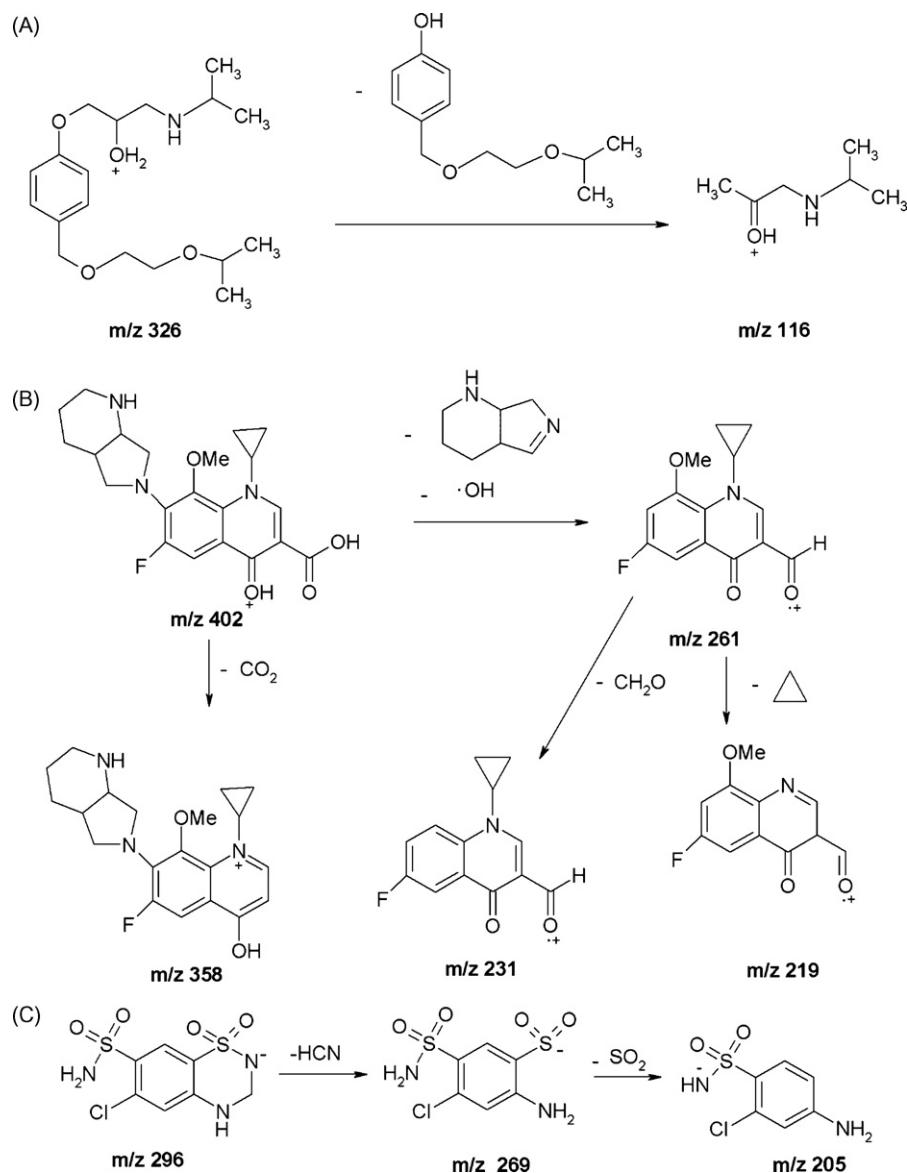

Fig. 3. Proposed fragmentation processes in the positive ion mode for (A) bisoprolol, (B) moxifloxacin (IS) and in negative ion mode for (C) hydrochlorothiazide.

Table 3
Analytical sensitivities (γ) for low, medium, and high quality control samples (QC$_L$, QC$_M$ and QC$_H$) of bisoprolol or hydrochlorothiazide.

	Bisoprolol			Hydrochlorothiazide		
	Concentration (ng/mL)	$S (\pm)^a$	Analytical sensitivity $(\gamma)^b$	Concentration (ng/mL)	$S (\pm)$	Analytical sensitivity $(\gamma)^b$
QC$_L$	0.300	0.025	2.672	3.00	0.278	0.071
QC$_M$	15.00	1.023	0.065	40.00	2.347	0.008
QC$_H$	24.00	1.271	0.053	64.00	2.451	0.008

[a] S: standard deviation.
[b] γ = slope/s.

Table 4
Summary of data pertaining to; instrument precision, inter-day and intra-day precision and accuracy.

	Bisoprolol			Hydrochlorothiazide		
Concentration (ng/mL)	0.30	15.00	24.00	3.00	40.00	64.00
						Instrument
Precision (CV %)	0.00	0.80	0.85	3.42	1.29	1.39
						Intra − day
Precision (CV %)	3.03	3.55	2.44	3.61	4.08	4.31
Accuracy (% RE)	10.0	−0.60	4.00	1.67	4.73	−2.47
						Inter − day
Precision (CV %)	6.45	5.97	6.49	8.06	7.58	5.84
Accuracy (% RE)	3.33	0.47	−0.08	3.33	0.92	0.66

CV % = SD of measured value/mean measured value × 100; % RE = (measured concentration − nominal concentration)/nominal concentration × 100.
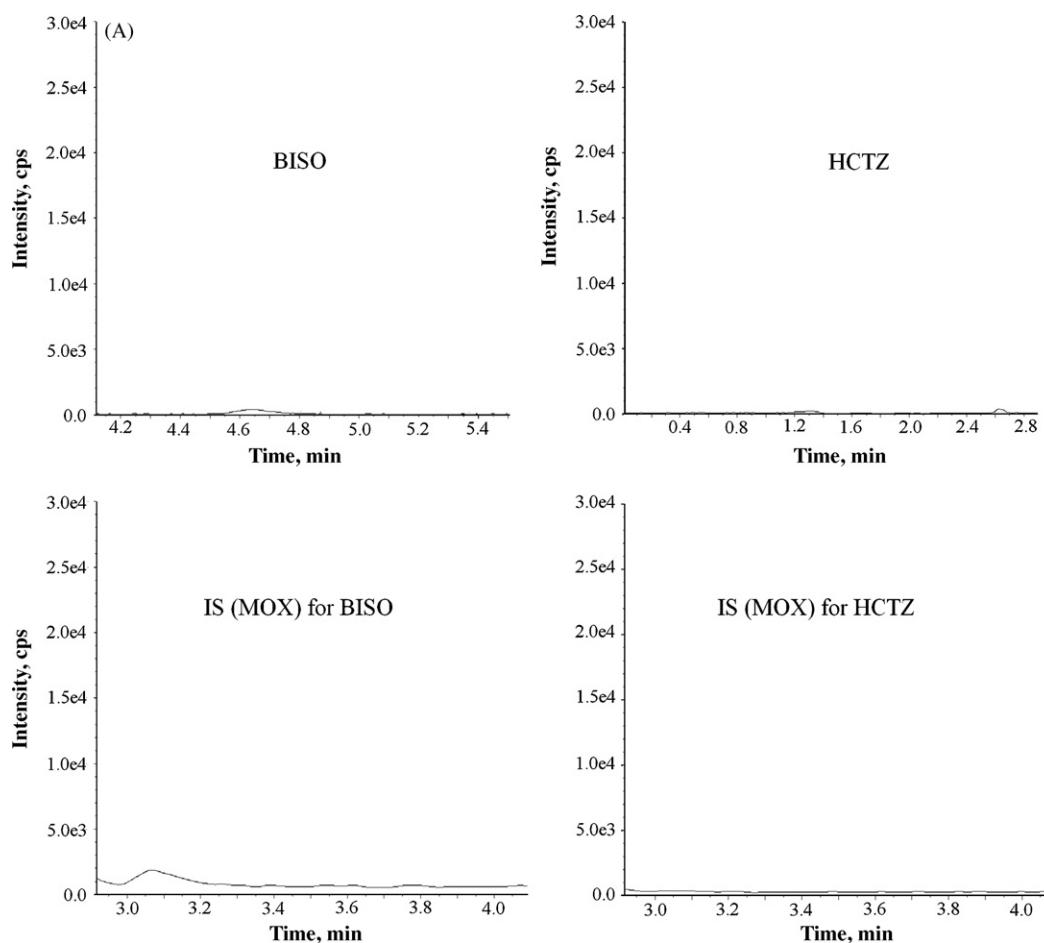
Fig. 4. Mass chromatograms for bisoprolol (BISO), hydrochlorothiazide (HCTZ) and the internal standard (IS) moxifloxacin (MOX) from human plasma extracts: (A) blank plasma sample, (B) a blank plasma sample spiked with bisoprolol (0.10 ng/mL), hydrochlorothiazide (1.00 ng/mL) and the IS (9.0 ng/mL), and (C) a volunteer plasma sample 2.33 h after an oral administration of a tablet containing 5 mg bisoprolol and 12.5 mg hydrochlorothiazide in fasting state.
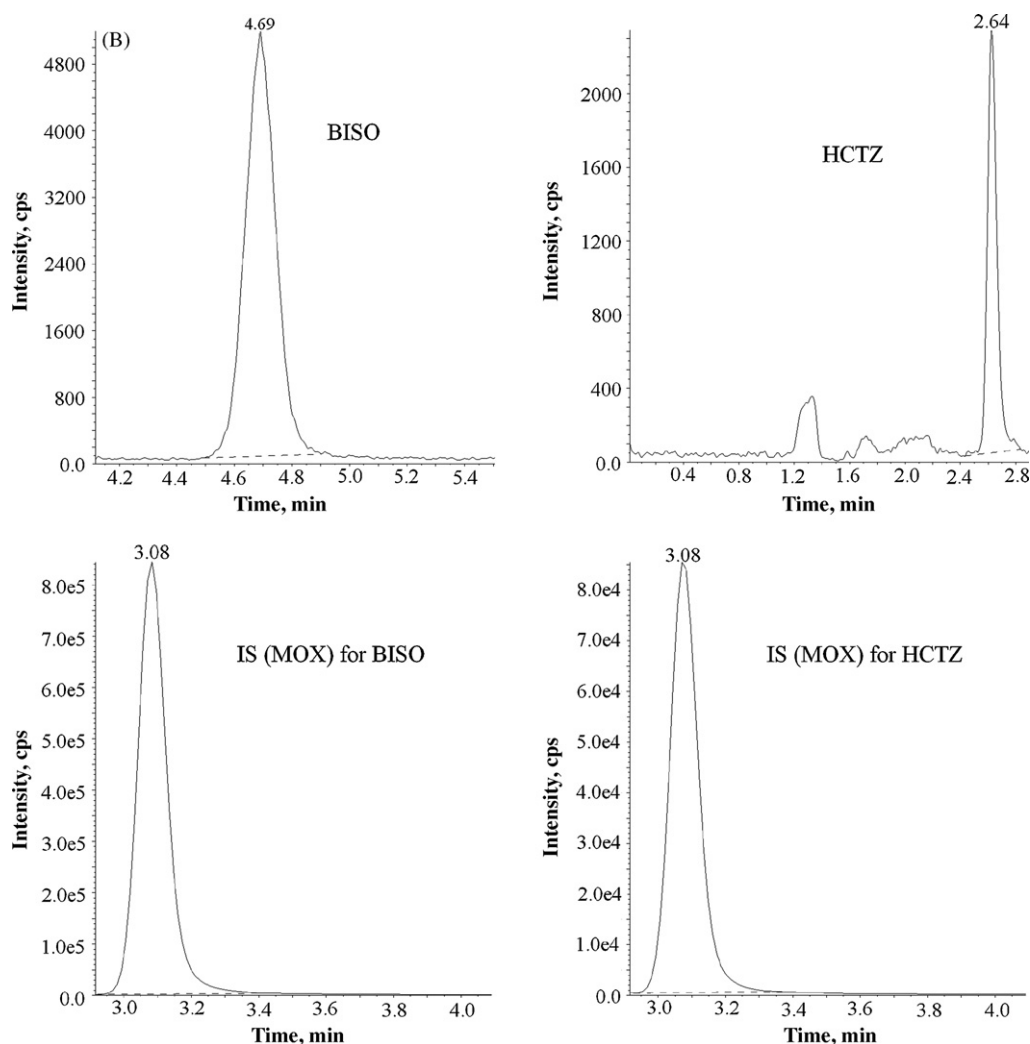
Fig. 4. (*Continued*)

symmetric peak shapes and short retention time for both ana-
lytes and the internal standard. A mobile phase composition of:
an ammonium acetate solution (1 mM) with formic acid (0.2%) -
methanol-acetonitrile (65:17.5:17.5, v/v/v (%)) was optimized, and
a flow rate was set at 0.65 mL/min. Acetonitrile was used for
protein precipitation, because of its good efficiency in precip-
itating and extraction. Moxifloxacin was chosen as an internal
standard because of its retention time which is similar to those of
analytes.

3.3. Method validation

3.3.1. Recovery

Absolute recovery was evaluated by comparing the peak areas
obtained from extracted spiked plasma standards with peak areas
from standards in the mobile phase. The mean absolute recovery of
BISO was 101.7, 94.41 and 90.70% for concentration levels of 0.30,
15.00 and 24.00 ng/mL. The mean absolute recovery for HCTZ was
36.77, 37.21 and 34.80% for concentration levels of 3.00, 40.00 and
64.00 ng/mL.

3.3.2. Linearity, linear working range and calibration models

Response functions from calibrators were recorded individually
for BISO and HCTZ. Each was plotted against the corresponding con-
centrations in the dynamic ranges of 0.10–30.0 (ng/mL) for BISO and
1.00–80.0 (ng/mL) for HCTZ.

Linearity was demonstrated both by visual inspection of calibra-
tion curves of concentration response and by the high correlation
coefficients of 0.9988 for BISO and 0.9994 for HCTZ.

Using the optimum weighted regression model with a statisti-
cal weight $1/x^2$, calibration equations of $y = 0.0668x + 0.0003$ and
$y = 0.0197x + 0.0044$ were obtained for BISO and HCTZ, respectively.

3.3.3. Sensitivity and limits of quantitation

Two types of sensitivity values were evaluated: (a) calibration
sensitivity, equated with the slope of the calibration graphs and (b)
analytical sensitivity (γ), $\gamma = b/s$, where b is the slope of the cali-
bration graph, and s is the standard deviation of the measurement.
The analytical sensitivity offers the advantage of being relatively
insensitive to amplification factors of the analytical signals and
independent of the measurement unit for s. its disadvantage is
that practically the analytical sensitivity is often concentration
dependent, since s vary with concentration [27]. The calibration
sensitivities were 0.0668 ($\pm$0.0071) and 0.0197 ($\pm$0.0019) for BISO
and HCTZ, respectively. Table 3 summarizes the results of analyti-
cal sensitivities for both analytes, since the standard deviation for
QCL was 0.025, it was reflected in the value of analytical sensitiv-
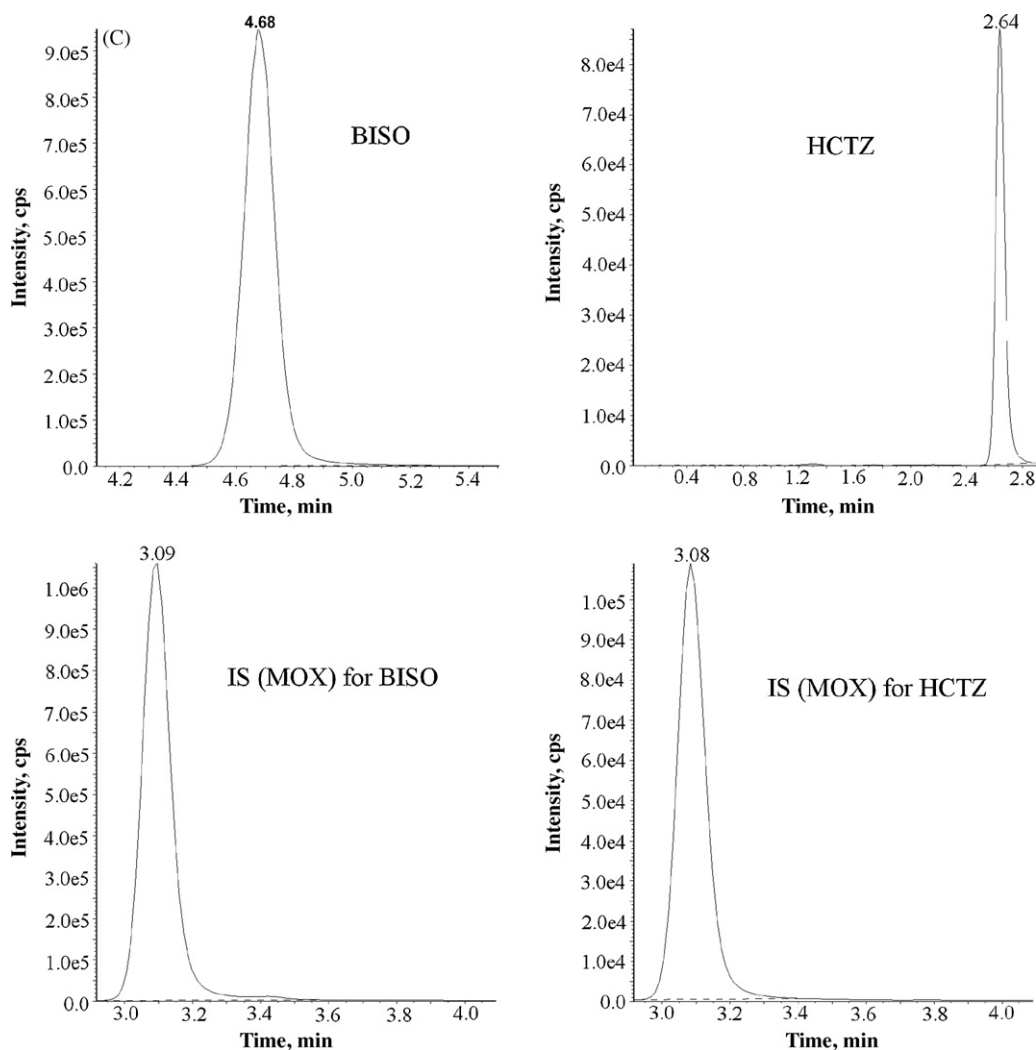ity (2.672) compared to 0.053 for QCH. The same observation was

Fig. 4. (*Continued*).

also demonstrated for the HCTZ as illustrated in Table 2. The LLOQ of BISO and HCTZ were found to be 0.10 and 1.00 (ng/mL), respectively.

3.3.4. Precision and accuracy

Ten replicate injections of the same standard mixture in the mobile phase, containing three different concentrations of both drugs and the IS, employed to determine instrument precision and accuracy. Six replicate measurements of each quality control matrix based standards for both analytes were chromatographed to evaluate method precision as well as to evaluate intra-day precision and accuracy. Inter-day precision and accuracy measured over fourteen days. The results are summarized in Table 4.

3.3.5. Selectivity and specificity

Six different sources of blank plasma were harvested under controlled conditions in a fasted state, extracted and analyzed using the developed method. No interferences were observed at the retention times of BISO, HCTZ and IS. Samples were compared with those obtained from an extract of a previously spiked plasma sample at the LLOQs containing both analytes and the IS. Representative chromatograms of the blank human plasma and blank human plasma spiked with BISO (0.10 ng/mL), HCTZ (1.00 ng/mL) and IS (9.0 ng/mL) and a plasma sample from a healthy volunteer 2.33 h after an oral administration of a tablet containing 5 mg BISO and 12.5 mg HCTZ are shown in Fig. 4.

Selectivity was also investigated with respect to caffeine and common OTC drugs including: diclofenac, theophylin, and acetaminophen. None of the investigated interferences showed analytical signal(s) at retention times of BISO, HCTZ or the IS.

3.3.6. Stability

Stability during sample collection, storage, and processing was investigated. Stability data was evaluated with respect to analytical signals obtained from freshly prepared QC samples.

Stability experiments extended throughout the analysis duration and assay of the last harvested sample. For short term stability studies, quality control including QCL and QCH were thawed and kept un-extracted at room temperature for 6, 12 and 24 h. Samples were then extracted and analyzed. Autosampler stability was evaluated over 24 h. Long term matrix based solution stability was investigated under prolonged storage conditions ($-86\,^{\circ}$C) for the study period. Freeze and thaw stability covered five freeze-thaw cycles. The data is summarized in Table 5; these results showed that, there is no significant degradation of either BISO or HCTZ was observed under the test conditions.

4. Application: pharmacokinetic study

The validated bioanalytical method was successfully applied for the determination of plasma concentrations of BISO and HCTZ in a

Table 5
Summary of stability data pertaining to bisoprolol and hydrochlorothiazide.

Storage condition	Drug	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	% Recovered
Short term stability (24 h in plasma)	Bisoprolol	0.30	0.27	90.0
		24.00	23.13	96.4
	Hydrochlorothiazide	3.00	2.95	98.3
		64.00	56.28	87.9
Autosampler stability after 24 h	Bisoprolol	0.30	0.32	106.7
		24.0	23.17	96.5
	Hydrochlorothiazide	3.00	3.17	105.7
		64.00	65.17	101.8
Freeze-thaw Cycles ($N = 5$)	Bisoprolol	0.30	0.31	103.3
		15.00	16.11	107.4
		24.00	25.50	106.3
	Hydrochlorothiazide	3.00	3.23	107.7
		40.00	39.93	99.8
		64.00	62.8	98.1
Long term stability (120 days, $-86\,^\circ$C)	Bisoprolol	0.30	0.30	100.0
		15.00	14.42	96.1
		24.00	23.06	96.1
	Hydrochlorothiazide	3.00	3.12	104.0
		40.00	41.03	102.6
		64.00	65.64	102.6

% Recovered = measured concentration/nominal concentration $\times$ 100.

pharmacokinetic study in 32 healthy male volunteers, who received a tablet containing 5 mg BISO and 12.5 mg HCTZ in a fasted state. Venous blood samples (8 mL) were collected into heparinized tubes at the following time points: immediately before dose administration (0.00) and at, 0.33, 0.67, 1.00, 1.33, 1.67, 2.00, 2.33, 2.67, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 8.00, 10.0, 12.0, 24.0, 36.0, 48.0 and 72.0 h after dosing. Blood samples were centrifuged (2000 rpm for 10 min); plasma was separated and stored at $-86\,^\circ$C until assay.

The mean pharmacokinetic profiles are illustrated in Fig. 5, whereas the pharmacokinetic parameters of C_{max}, T_{max}, $t_{1/2}$, $AUC_{0-72\,h}$, and $AUC_{0-\infty}$ are summarized in Table 6.

Table 6
Summary of the pharmacokinetic parameters for a tablet containing 5 mg bisoprolol and 12.5 mg hydrochlorothiazide.

Parameter	Bisoprolol	Hydrochlorothiazide
C_{max} (ng/mL)	23.37 (4.31)	62.38 (15.92)
T_{max} (h)	2.07 (1.20)	2.01 (0.88)
$t_{1/2}$ (h)	10.81 (1.54)	9.85 (2.81)
$AUC_{0-72\,h}$ (ng h/mL)	372.47 (69.67)	423.82 (98.88))
$AUC_{0-\infty}$ (ng h/mL)	379.89 (70.38)	450.98 (98.85)

5. Conclusion

A selective, sensitive, precise and accurate LC–MS/MS method for the simultaneous determination of BISO and HCTZ in human plasma has been developed, optimized and validated. A limit of quantitation of 0.10 and 1.00 ng/mL for BISO and HCTZ enabled an accurate evaluation of pharmacokinetic parameters after dosing with bisoprolol and hydrochlorothiazide. The method has been successfully applied to the pharmacokinetic study of 5 mg BISO and 12.5 mg HCTZ tablet in healthy volunteers.
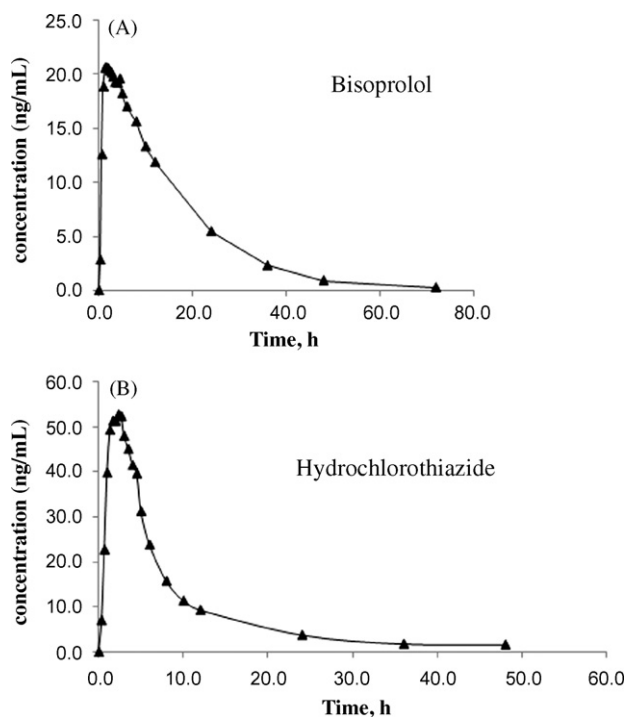
Acknowledgments

Fig. 5. Mean plasma concentration-time profiles for (A) bisoprolol and (B) hydrochlorothiazide for the 5 mg bisoprolol and 12.5 mg hydrochlorothiazide tablet formulations administrated to 32 healthy volunteers in the fasting state.

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