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Simultaneous determination of metoprolol succinate and amlodipine besylate in human plasma by liquid chromatography–tandem mass spectrometry method and its application in bioequivalence study

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ABSTRACT

A simple, sensitive and specific liquid chromatography–tandem mass spectrometry method was developed and validated for quantification of metoprolol succinate (MPS) and amlodipine besylate (AM) using hydrochlorothiazide (HCTZ) as IS in human plasma. Both the drugs were extracted by simple liquid–liquid extraction with chloroform. The chromatographic separation was performed on a reversed-phase peerless basic C18 column with a mobile phase of methanol–water containing 0.5% formic acid (8:2, v/v). The protonated analyte was quantitated in positive ionization by multiple reaction monitoring with a mass spectrometer. The method was validated over the concentration range of 1–100 ng/ml for MPS and 1–15 ng/ml AM in human plasma. The MRM transition of *m/z* 268.10–103.10, *m/z* 409.10–334.20 and *m/z* 296.00–205.10 were used to measure MPS, AM and HCTZ (IS), respectively. This method was successfully applied to the pharmacokinetic study of fixed dose combination (FDC) of MPS and AM formulation product after an oral administration to Indian healthy human volunteers.

1. Introduction

Metoprolol is a beta$_1$-selective (cardioselective) adrenergic receptor blocking agent. This preferential effect is not absolute, however, and at higher plasma concentrations, metoprolol also inhibits beta$_2$-adrenoreceptors, chiefly located in the bronchial and vascular musculature. Metoprolol has no intrinsic sympathomimetic activity, and membrane-stabilizing activity is detectable only at plasma concentrations much greater than required for beta-blockade [1,2].

Amlodipine besylate is the besylate salt of amlodipine, a long-acting calcium channel blocker. Amlodipine is a dihydropyridine calcium antagonist (calcium ion antagonist or slow-channel blocker) that inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle. Amlodipine is a peripheral arterial vasodilator that acts directly on vascular smooth muscle to cause a reduction in peripheral vascular resistance and reduction in blood pressure [3].

Additive reductions in heart rate, cardiac conduction, and cardiac contractility may occur when calcium channel blockers are used concomitantly with beta blockers, particularly in patients with ventricular or conduction abnormalities. While this combination may be useful and effective in some situations, potentially serious cardiovascular adverse effects such as congestive heart failure, severe hypotension, and/or exacerbation of angina may occur. The proposed mechanisms include additive slowing in AV conduction, reduced cardiac contractility secondary to beta-blockade, and decreased peripheral vascular resistance secondary to calcium channel blockade. In addition, some calcium channel blockers may inhibit the CYP450 metabolism of hepatically metabolized beta blockers, resulting in increased serum concentrations [3]. Therefore, fixed dose combinations (FDCs) remain the first choice when they are available. Co blistered combinations (CBCs) are the second choice. Single products are third but least desirable. This therapy has the benefits of slowing resistance, improving clinical outcomes, and facilitating logistics. So an analytical method for the simultaneous determination of both the analytes in plasma was necessary. Fig. 1 shows the chemical structure of MPS, AM and HCTZ (IS) used in this study. Literature survey reveals few analytical methods for the determination of AM viz. HPLC [4–6], LC–MS/MS [7-8], UPLC [9]. Determination of MPS viz. HPLC [5,10-15], LC–MS [16–19], GC–MS [20,21] has been reported. Enantiomers of MPS have also been determined by HPLC [22–24]. Usually in a clinical study, large

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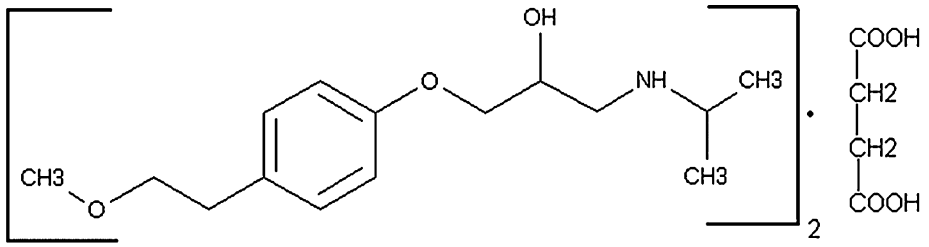
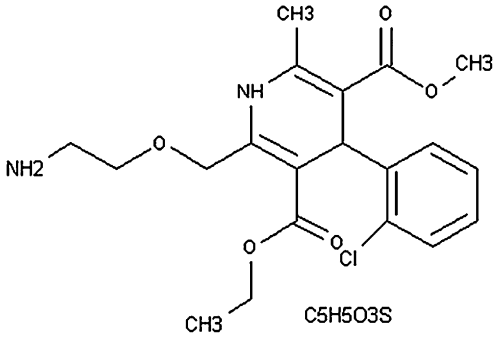
(A)



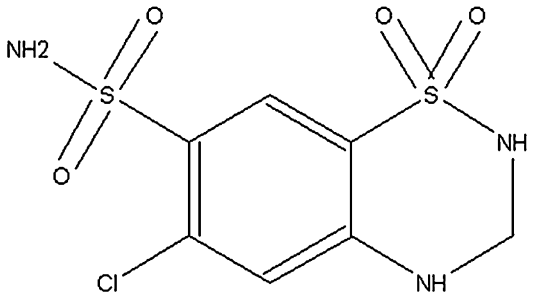
(±)1-(isopropylamino)-3-[p-(2-methoxyethyl) phenoxy]-2-propanol succinate (2:1) (salt)

(B)



3-Ethyl-5-methyl (±)-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate, monobenzenesulphonate

(C)



6-chloro-3,4-dihydro-2*H*-1,2,4-benzo-thiadiazine-7-sulfonamide 1,1-dioxide

Fig. 1. Chemical structure of (A) MPS, (B) AM and (C) IS (HCTZ).

numbers of samples are collected. So a rapid and reliable assay method is essential to analyze such huge pool of samples in a very short time. An ideal method should have simple sample preparation and adequate sensitivity. Liquid chromatography coupled with mass spectrometry (LC–MS/MS) is one such efficient analytical tool which meets most of the above needs [25] particularly in simultaneous analysis of fixed dose combination dosage forms. LC–MS/MS method facilitates analyzing large samples in a very short period of time. Previously reported methods [4–24] either had long retention time (10–15 min) or suffered from low sensitivity and in some cases required large sample injection volumes (100 μl). There are various methods reported for the determination of MPS [5,10–24] and AM [4–9] separately. To the best of our knowledge, there is no method reported in the literature for the simultaneous determination of these analytes by LC–MS/MS. Hence the main objective of this work was to develop a simple, sensitive rapid and reliable mass spectrometry (LC–MS/MS) method for the simultaneous quantification of MPS and AM in human plasma.

2. Experimental

2.1. Chemicals and reagents

MPS, AM and HCTZ (IS) were obtained from Madras Pharmaceuticals, Chennai-600096. HPLC grade formic acid and methanol were purchased from Merck (Mumbai, India) and Tris buffer, EDTA and chloroform were also purchased from Merck (Mumbai, India). HPLC grade water generated from Milli Q water purification system was used throughout the analysis.

2.2. Instrumentation

The liquid chromatographic system consisting of Shimadzu series LC 20 AT pump, CTO 10 AS VP column oven, SIL 20 AC auto-sampler (Kyoto, Japan) were used for the separation. The LC–MS/MS system (API 2000) with triple quadrupole tandem mass spectrometer (AB Sciex Instruments, Foster, Canada) was used for quantitative determination of MPS and AM in human

Table 1
Main working parameters for mass spectrometry

Parameter	Value
Curtain gas (CUR) (psi)	10.00
Ion spray voltage (IS) (V)	5500.00
Source gas temperature (TEM) (°C)	375.00
Ion source gas (Gas 1) (psi)	55.00
Heater gas 2 (Gas 2) (psi)	30.00
Collision associated dissociation (CAD gas) (psi)	10.00

Parameter	MPS	AM	HCTZ (I.S)
Declustering potential (DP) (V)	25.00	35.00	28.00
Focussing potential (FP) (V)	380.00	330.00	395.00
Entrance potential (EP) (V)	9.00	3.60	10.00
Collision energy (CE) (V)	55.00	35.00	35.00
Collision cell exit potential (CXP) (V)	5.00	10.00	5.00
Mode of analysis	(+)ve	(+)ve	(−)ve
Ion transition *m/z*	268.10 → 103.10	409.10 → 334.20	296.0 → 205.10

plasma.Data acquisition was performed with Analyst 1.4.1. software.

2.3. Chromatographic conditions

Chromatographic separation was achieved on a Peerless basic C18 column, 33 mm × 4.6 mm, particle size 5 μm (Chromatopak, India). Mobile phase used for separation of the analytes was methanol:water containing 0.5% formic acid (8:2, v/v). The flow rate was set at 1 ml/min. The injection volume was 25 μl and the total run time was 3 min. The column was maintained at ambient temperature (24 °C) whilst the autosampler temperature was set at 10 °C.

2.4. Mass spectrometry

Electrospray ionization (ESI) with multiple reaction monitoring (MRM) was used to acquire the mass spectra of the compounds. Ions were measured in positive and negative ionization mode. The tuning parameters were optimized by injecting 100 ng/ml of standard solution containing all three drugs at 25 μl/min by means of an external syringe pump directly connected to the mass spectrometer. The turbo ion spray source temperature was set at 375 °C and the turbo ion spray voltage was set at 5500 V. The nebulizer gas (GS1), the turbo ionspray gas (GS2) and the curtain gas values were set at 55, 30 and 10 psi, respectively. The collision associated dissociation (CAD) gas value was fixed at 10 (arbitrary units). Optimized values of compound related parameters and source gas parameters are summarized in Table 1.

2.5. Standard solutions

Separate solutions containing 1 mg/ml of MPS, AM and HCTZ (IS) were prepared using mobile phase, respectively. These solutions were further diluted suitably with the mobile phase to obtain a stock solution of 1 μg/ml. The stock solutions prepared for the drugs were diluted further to obtain eight working solutions for calibration standards. All solutions were stored at 2–8 °C.

2.6. Calibration curves

A eight point standard calibration solutions of MPS and AM were prepared by spiking blank plasma with appropriate amounts of analytes in human plasma to yield final concentrations of 1.5, 3, 5, 10, 25, 50, 75 and 100 ng/ml for MPS and 1.25, 2, 2.5, 3.5, 5, 7, 10 and 12.5 ng/ml for AM. Three quality control (QC) samples were prepared at three concentration levels of 7.5, 50 and 90 ng/ml for MPS and 3, 7 and 12 ng/ml for AM. Calibration curves were plotted with peak area ratio of drug and IS on *Y*-axis and concentration on *X*-axis.

2.7. Sample preparation and extraction

Liquid–liquid extraction procedure was used for the extraction of the drug from the plasma. Calibration standards, quality control samples were treated with 5 ml chloroform. 100 μl of IS (100 ng/ml) and 250 μl of Tris buffer were added with each 1 ml plasma sample and vortex mixed for 10 min followed by centrifugation for another 10 min. The organic layer containing the analytes was separated, transferred to a separate test tube and evaporated to dryness under a stream of N_2 at 40 °C. The residue obtained on drying was reconstituted with the 150 μl of mobile phase. The reconstituted sample was transferred to an auto sampler vial and injected into the liquid chromatography mass spectrometry (LC–MS/MS) system.

2.8. Method validation

The accuracy, sensitivity, precision, stability, recovery, reproducibility and reliability of the analytical method were confirmed by validation in accordance with the USFDA guidelines [28].

2.8.1. Linearity and LLOQ

To establish linearity, a series of calibration standards were prepared by adding a known concentration of MPS, AM and HCTZ (IS) to drug free human plasma and analyzed. The lowest concentration on the standard curve with detector response five times greater than the drug free (blank) human plasma was considered as the LLOQ. The analyte peak in LLOQ sample should be identifiable, discrete and reproducible with a precision of 20% and an accuracy of 80–120%.

2.8.2. Specificity

Specificity is the ability of an analytical method to differentiate and quantify the analyte in presence of other components in the sample. The specificity of the method was evaluated by screening six lots of blank plasma. These lots were spiked with known concentration of analytes along with IS at low, medium and high concentrations. The spiked samples were analyzed after extraction to confirm lack of interference and absence of lot-to-lot variation.

2.8.3. Accuracy and precision

Inter-day precision and accuracy of the assay was evaluated by running three validation batches on three separate days. Each batch consisted of one set of calibration standards and five replicates of

quality control (QC) samples at low, medium and high concentration. The intra-day precision and accuracy was also consisted of one set of calibration standards and five replicates of quality control (QC) samples at low, medium and high concentration. A comparison was made between the obtained values and the experimental values. Precision was expressed as percentage of relative standard deviation (% R.S.D.). The mean value of accuracy should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The precision determined at each concentration level should not exceed 15% of R.S.D. except for the LLOQ, where it should not exceed 20% of R.S.D.

2.8.4. Extraction recovery and matrix effect

The extraction recovery of the analytes from the plasma was evaluated by comparing the mean detector responses of three replicates of processed QC samples at low, medium high concentrations to the detector responses of standard solutions of same concentration. Recovery of an analyte need not be 100%, but the extent of recovery of analyte and the IS should be consistent, precise and reproducible [26]. Endogenous matrix components may change the efficiency of droplet formation or droplet evaporation, which in turn affects the amount of charged ion in the gas phase that ultimately reaches the detector [27–29]. Two sets of samples were prepared by directly spiking the analytes into reconstitution solution with and without the presence of residue extracted from control plasma. Ion suppression was assessed at three QC sample concentrations by comparing the mean analyte peak areas obtained from these two sets of testing samples. Matrix effect was calculated [16] as per the following equation:

$$\text{matrix effect} = \left(\frac{\text{analyte peak area of extracted plasma residue}}{\text{analyte peak area of neat solution}}\right) \times 100$$

2.8.5. Stability

2.8.5.1. Long and short term stability. Three aliquots of each low and high QC samples were kept in deep freezer at $-20\,^{\circ}C \pm 5\,^{\circ}C$ for 1 month. The samples were processed and analyzed and the concentrations obtained were compared with the actual value of QC samples to determine the long term stability of analyte in human plasma. Three aliquots each of high and low unprocessed QC samples were kept at ambient temperature (20–30 °C) for 8 h in order to establish the short term stability of the analytes. The samples were analyzed and the concentrations obtained were compared with the actual values of QC samples. Samples were concluded stable if the % R.S.D. of the stability samples was within ±15% of the actual value.

2.8.5.2. Post-preparative stability. Three aliquots each of high and low QC samples were stored at 10 °C in an auto sampler for 24 h, analyzed and the concentrations were compared with the actual values. Stability was concluded when the % R.S.D. was within ±15% of the actual value.

2.8.5.3. Stock solution stability. Separate standard stock solutions containing 100 ng/ml of MPS, AM and IS were prepared and stored at 2–8 °C for 30 days. The response obtained from the three drugs was calculated and compared with that of the freshly prepared solutions of the same concentration with an acceptable limit of ±2% [30].

2.8.5.4. Freeze and thaw stability. The stability of the analytes after three freeze and thaw cycles was determined at low, high QC samples. The samples were stored at −20 °C for 24 h and thawed unassisted at room temperature. After completely thawing, the samples were refrozen for 12–24 h. After three freeze–thaw cycles, the concentration of the samples were analyzed.

2.8.5.5. Dry state stability. Three aliquots each of low and high QC samples were stored at −20 °C without reconstitution after extraction (i.e. in dry state). The samples were analyzed after 24 h and a deviation of ±15% was acceptable.

2.9. Application of the LC–MS/MS method

The LC–MS/MS method was successfully applied to the pharmacokinetic study of FDC of MPS and AM in healthy human volunteers. Twelve healthy human volunteers aged 21–27 years were admitted in CPU (Clinical Pharmacological Unit) of Bioequivalence Study Centre (Dept. of Pharm Tech., Jadavpur University, Kolkata, India). After an overnight fast (12 h), each volunteer was given either single dose of test preparation (FDC tablet containing 50 mg of metoprolol succinate as extended release and 5 mg of amlodipine besylate) or reference preparation tablet Met XL AM* 50 (FDC tablet containing metoprolol succinate 50 mg ER and amlodipine besylate 5 mg) with 240 ml of water. Their TPR, BP was recorded and an indwelling intravenous catheter was introduced with strict aseptic precautions in the suitable vein for blood collection. A total of 14 blood samples were collected at 0 h (before drug administration) and 1.0, 2.0, 4.0, 4.5, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 12.0, 24.0, 48.0 and 120.0 h (after drug administration) in the test tubes with EDTA at each time point. Breakfast, lunch and dinner were provided after 3, 6, and 13 h respectively after drug ingestion. Collected blood samples were centrifuged immediately at 10,000 rpm in cool centrifuger, separate the plasma and stored frozen at −20 °C until analysis with appropriate labeling of volunteer code no., study date and collection time.

3. Results and discussion

3.1. Internal standard

A stable analyte has to be used as an IS to deal with sample matrix effects. Since such IS is not available commercially, an alternative approach has been used. IS chosen should match the chromatographic properties, recovery and ionization properties of the analyte [32]. Hydrochlorothiazide was found to match these criteria and also serve our purpose of method development, therefore was chosen as an IS. Hydrochlorothiazide was selected because of its high recovery, the results (Table 4) indicate that the component of IS did not alter or deteriorate the performance of the proposed method, there are plenty of FDC combinations of MPS and HCTZ [2,35–37] and FDC of AM and HCTZ [38] indicates that there are not any drug–drug interaction and also the intensity of MPS and AM molecular ion peaks in mass spectrometric analysis remained unaffected as compared to others. Good chromatographic separation was another reason for its selection.

3.2. LC–MS/MS analysis

LC–MS/MS was employed for the simultaneous quantification of MPS and AM in human plasma. To the best of our knowledge there is no LC–MS/MS method reported for the simultaneous determination of these drugs in human plasma. The LC–ESI-MS/MS in MRM mode provided a highly selective method for the simultaneous determination of MPS and AM in human plasma. ESI source provided a better ionization of the compounds as compared to the atmospheric chemical ionization (APCI). The positive mode of ionization was selected for MPS and AM because the intensity of the molecular ion peaks was more in positive mode. The negative mode

of ionization was selected for IS (HCTZ), because the intensity of the molecular ion peaks was more in negative mode. In ion switching method we have selected one period and two experiments where 1st experiment is in positive ion mode and 2nd experiment is in negative ion mode and both the experiments will run parallely in a single period. This method also provides us a good result as we have applied this method to a bioequivalence study of fixed dose combination [34]. The positive product ion mass spectra of the molecular ions of MPS, AM and negative product ion mass spectra of the molecular ions of HCTZ are shown in Fig. 2. Quantitation of analytes in human plasma was based on the detector response ratio of analytes to IS. Total run time set for the samples tested was 3 min as shown in Fig. 3. The results indicated that a run time of 1.5 min was sufficient for sample analysis. On repeated injection of the samples,

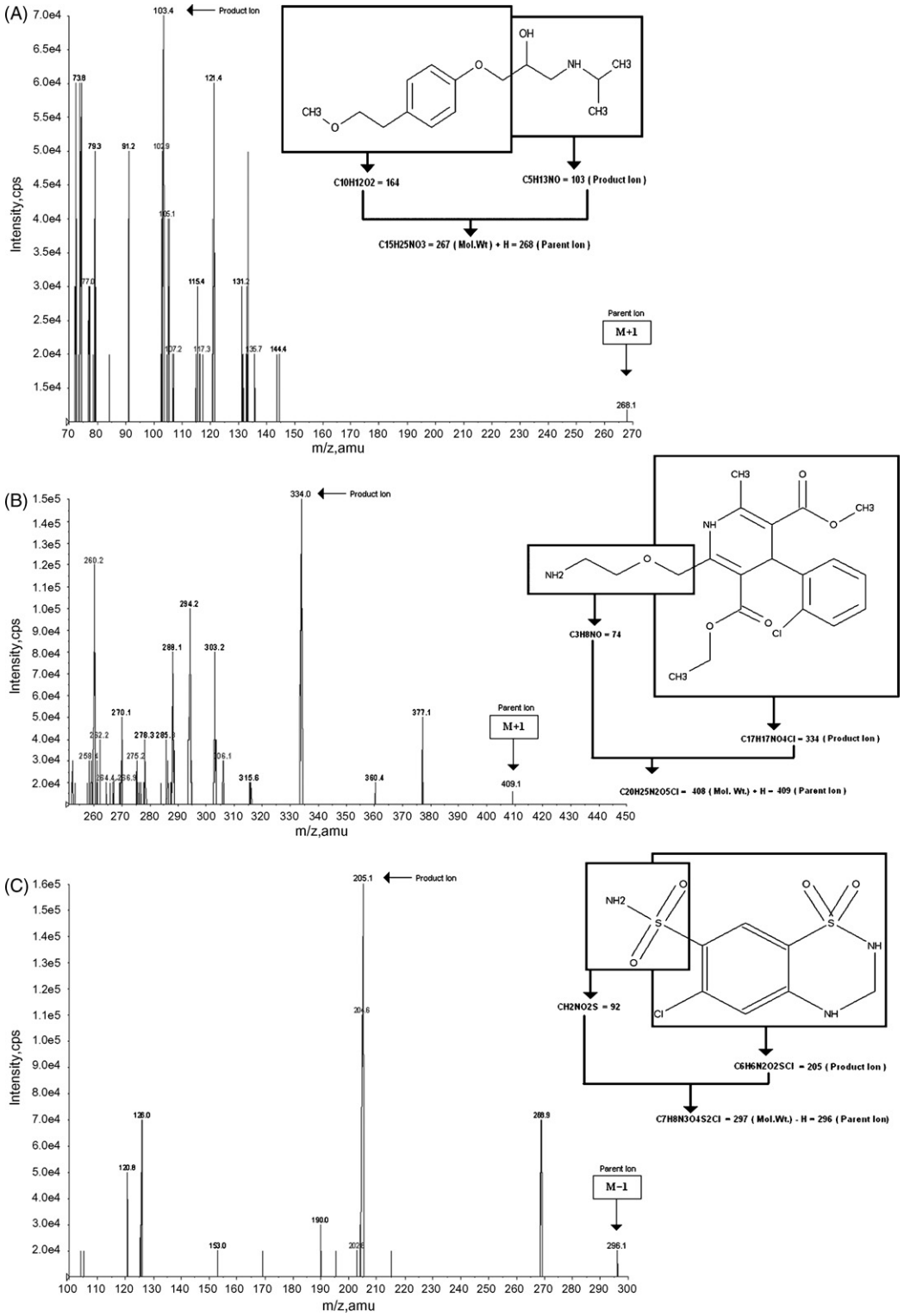


Fig. 2. Profile mass spectral data of (A) metoprolol [M+H]; (B) amlodipine [M+H]; and (C) hydrochlorothiazide [M−H] (IS) with their fragmentation interpretation.

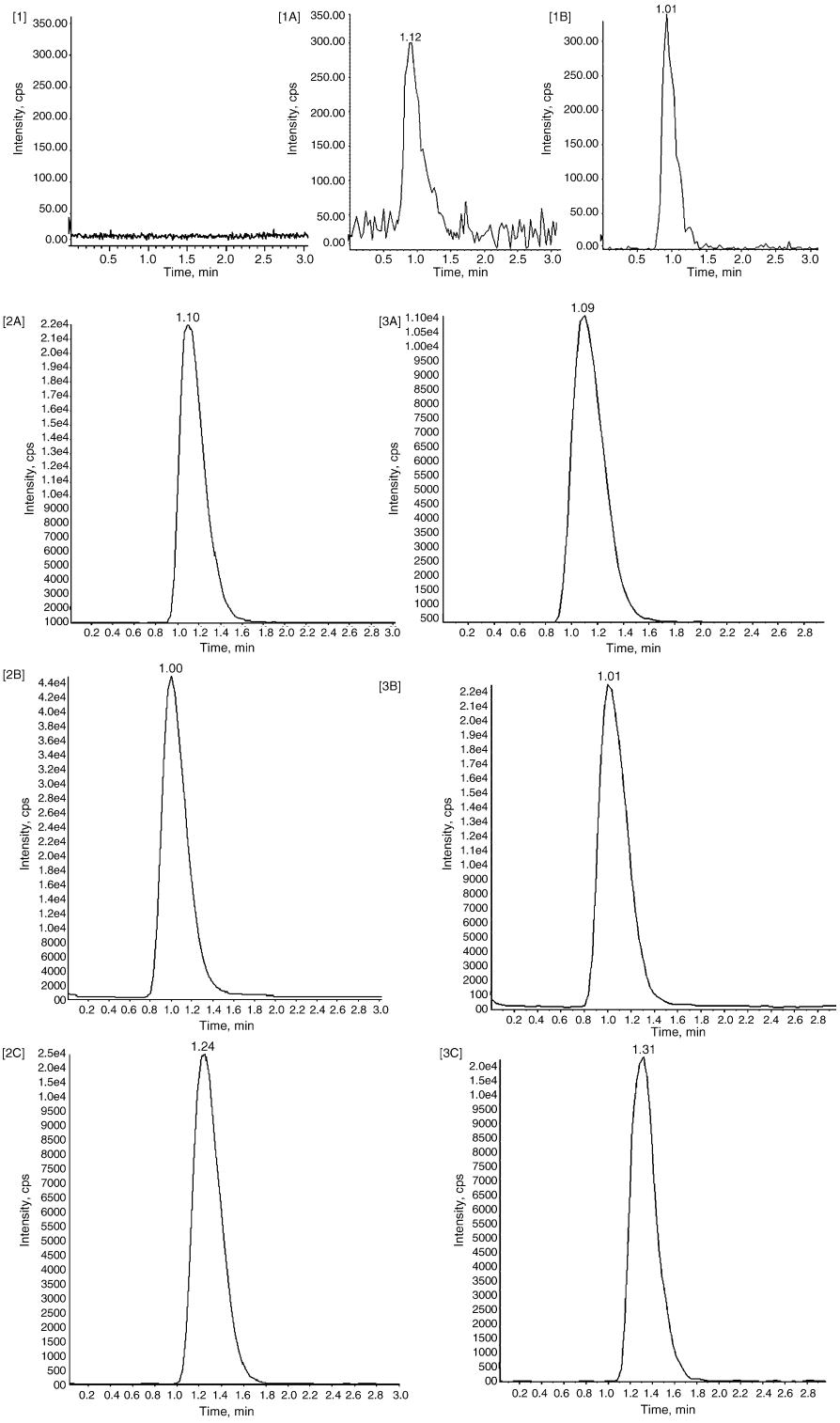


Fig. 3. Representative chromatograms of (1) blank plasma, (1A) LLOQ of MPS, (1B) LLOQ of AM (2) blank plasma spiked with (2A) MPS (50 ng/ml) (2B) AM (10 ng/ml) and (2C) IS (HCTZ 100 ng/ml) respectively and (3) volunteer plasma after oral administration of FDC, where (3A) MPS (3B) AM and (3C) IS (HCTZ 100 ng/ml), respectively.

the retention time never shifted beyond 10–15 s. MPS, AM and IS (HCTZ) were eluted at retention times of 1.0, 1.10 and 1.24 min, respectively (Fig. 3). The main analytes MPS and AM were separated with good resolution. Chromatographic separation of MPS, AM and IS (HCTZ) were not achieved under the set analytical conditions. However due to high selectivity of tandem mass spectra, complete chromatographic separation is not necessary any more [31]. The main advantage of this method is that a relatively large number of samples can be analyzed in short time thus increasing the output.

3.3. Linearity

The calibration curves were found to be linear over a range of 1.5–100 ng/ml for MPS and 1.25–12.5 ng/ml for AM. The average

Table 2
LOD, LOQ and calibration results

Analyte	Regression equation (ng/ml)	R^2	LOD (ng/ml)	LOQ (ng/ml)	Range
MPS	$y = 1.3925x - 0.3744$	0.9992	1	1.5	1.5–100
AM	$y = 0.8186x - 0.0534$	0.9988	0.5	1.25	1.25–12.5

Table 3
Intra- and inter-day precision and accuracy for MPS and AM ($n = 6$)

Analyte	QC sample (ng/ml)	Intra-day variation			Inter-day variation		
		Mean ± S.D.	R.S.D.%	Accuracy%	Mean ± S.D.	R.S.D.%	Accuracy%
MPS	7.5	7.12 ± 0.24	3.37	94.93	7.61 ± 0.45	5.91	101.47
	50	50.1 ± 1.45	2.89	100.20	49.25 ± 2.89	5.87	98.50
	90	89.59 ± 3.45	3.85	99.54	90.31 ± 6.14	6.80	100.34
AM	3	2.78 ± 0.12	4.32	92.67	2.95 ± 0.23	7.80	98.33
	7	6.59 ± 0.32	4.86	94.14	6.88 ± 0.59	8.58	98.29
	12	11.82 ± 0.58	4.91	98.50	12.03 ± 0.95	7.90	100.25

S.D. = standard deviation. % Relative standard deviation (R.S.D.) = (standard deviation/mean) × 100.

correlation coefficients obtained were 0.9992 and 0.9988 for MPS and AM, respectively. Table 2 summarizes the LOD LOQ and results of calibration. The LLOQ was found to be 1.5 ng/ml for MPS and 1.25 ng/ml for AM as shown in Fig. 3 and LOD was found to be 1 ng/ml for MPS and 0.5 ng/ml for AM.

3.4. Specificity

The specificity of the method was investigated by comparing chromatograms of six different sources of human plasma. No significant peaks were observed at the retention times of MPS, AM and IS (HCTZ) in human blank plasma. Representative chromatograms of blank plasma, blank plasma spiked with MPS, AM, IS (HCTZ) are shown in Fig. 3.

3.5. Accuracy and precision

Table 3 summarizes the mean values of accuracy and precision for both intra- and inter-day assays. Both precision and accuracy were within the acceptable ranges for bioanalytical purpose. Intra-day precision ranged from 2.89% to 3.85% for MPS and 4.32% to 4.91% for AM. Inter-day precision ranged from 5.87% to 6.80% for MPS and 7.80% to 8.58% for AM. The percentage of accuracy was in the range of 94.93–101.47% for MPS and between 92.67% to 100.25% for AM. The assay method demonstrated high degree of accuracy and precision.

3.6. Extraction recovery and matrix effect

Recovery results [16] presented that the maximum recovery was achieved with MPS (99.06%) followed by AM (77.68%). HCTZ extraction recovery ranged from 68.85% to 71.51%. The extraction recovery was found to be satisfactory as it was consistent, precise and reproducible. Thus single step liquid–liquid extraction procedure used in this method proved to be efficient and simple enough to extract three drugs (including IS) simultaneously from human plasma.

The endogenous components are mainly the cause of ion suppression effects during electrospray ionization. The extent of this effect is mainly dependent on sample extraction procedure and is also compound dependent [33]. The results indicated that the matrix components did not alter or deteriorate the performance of the proposed method as the % relative standard deviation (R.S.D.) of three QC samples was less than 3.37%, 4.91% and 4.67% for MPS,

Table 4
Extraction recovery and matrix effect of MPS, AM and HCTZ (I.S.) ($n = 6$)

Analyte	QC sample (ng/ml)	R.S.D.%	ER%	ME%
MPS	7.5	3.37	98.93	98.42
	50	2.89	97.20	99.06
	90	3.85	99.06	99.59
AM	3	4.32	73.38	98.88
	7	4.86	75.70	98.09
	12	4.91	77.68	99.15
HCTZ	100	4.67	71.51	100.05

S.D. = standard deviation. % Relative standard deviation (R.S.D.) = (standard deviation/mean) × 100. ER% = % extraction recovery. ME% = % matrix effect. matrix effect $= \left(\frac{\text{analyte peak area of extracted plasma residue}}{\text{analyte peak area of neat solution}}\right) \times 100$

Table 5
Stability summary of MPS and AM ($n = 6$)

Stability	QC sample (ng/ml)	MPS		
		Mean ± S.D.	R.S.D.%	Accuracy%
Long term (30 days)	7.5	6.8 ± 0.86	12.65	90.67
	90	85.97 ± 8.67	10.08	95.52
Short term (8 h)	7.5	7.38 ± 0.25	3.39	98.40
	90	89.57 ± 3.45	3.85	99.52
Post-preparative (24 h)	7.5	7.12 ± 0.37	5.20	94.93
	90	88.45 ± 2.83	3.20	98.28
Freeze–thaw	7.5	7.18 ± 0.34	4.74	95.73
	90	89.73 ± 4.62	5.15	99.70
Dry extract (24 h)	7.5	7.09 ± 0.72	10.16	94.53
	90	87.89 ± 7.46	8.49	97.66
Stability	QC sample	AM		
		Mean ± S.D.	R.S.D.%	Accuracy%
Long term (30 days)	3	2.78 ± 0.22	7.91	92.67
	12	11.34 ± 1.12	10.93	94.50
Short term (8 h)	3	2.85 ± 0.25	8.77	95.00
	12	11.67 ± 1.1	2 9.60	97.25
Post-preparative (24 h)	3	2.79 ± 0.31	11.11	93.00
	12	11.69 ± 1.41	12.06	97.42
Freeze–thaw	3	2.89 ± 0.29	10.03	96.33
	12	11.29 ± 1.34	11.87	94.08
Dry extract (24 h)	3	2.77 ± 0.26	9.39	92.33
	12	10.98 ± 0.59	5.37	91.50

S.D. = standard deviation. % Relative standard deviation (R.S.D.) = (standard deviation/mean) × 100.

Table 6
Pharmacokinetic parameters for a single FDC of MPS 50 mg as extended release and AM 5 mg tablet after oral administration to 12 Indian healthy human volunteers

Pharmacokinetic parameter	MPS		AM	
	Reference, mean ± S.D.	Test, mean ± S.D.	Reference, mean ± S.D.	Test, mean ± S.D.
C_{max} (ng/ml)	52.50 ± 4.34	50.84 ± 3.65	7.08 ± 0.74	6.83 ± 0.36
T_{max} (h)	6.16 ± 0.77	6.33 ± 0.57	6.75 ± 0.62	6.91 ± 0.70
AUC_{0-t} (ng h/ml)	514.82 ± 30.60	509.26 ± 35.91	244.08 ± 31.30	243.10 ± 67.16
$AUC_{0-\infty}$ (ng h/ml)	546.75 ± 35.01	541.46 ± 43.14	359.21 ± 41.21	379.43 ± 70.19
$t_{1/2}$ (h)	5.06 ± 0.49	5.07 ± 0.47	33.03 ± 2.84	34.93 ± 1.48
K_{el} (h^{-1})	0.138 ± 0.013	0.137 ± 0.012	0.021 ± 0.002	0.020 ± 0.001
Relative bioavailability (%)	100	98.92	100	99.60

AM and IS respectively indicates the reproducibility of peak area as well as the extracts were 'clean' and no unseen component interfere with the ionization of the analytes. The matrix effect on the estimation of the analytes was shown in Table 4.

3.7. Stability

Table 5 summarizes the results of stability study carried out under various conditions. Both the analytes were found to be stable at ambient temperature (20–30 °C) for at least 8 h in human plasma. The percentage of accuracy obtained was more than 90.67% and 91.50% for MPS and AM, respectively. The LQC and HQC samples of both the analytes remained unaffected at −20 °C for 1 month. In an autosampler maintained at 10 °C, plasma samples of MPS and AM were stable for more than 24 h. The freeze–thaw stability results showed that MPS and AM are stable for at least three freeze–thaw cycles. Stability results indicated that human plasma samples could be thawed and refrozen without compromising the integrity of the samples. Extracted and dried residues were stable for 24 h without any change in the concentration. Working solutions of MPS, AM and IS were stable and the deviation was less than ±2%. There was no much degradation in the solutions even after 30 days.

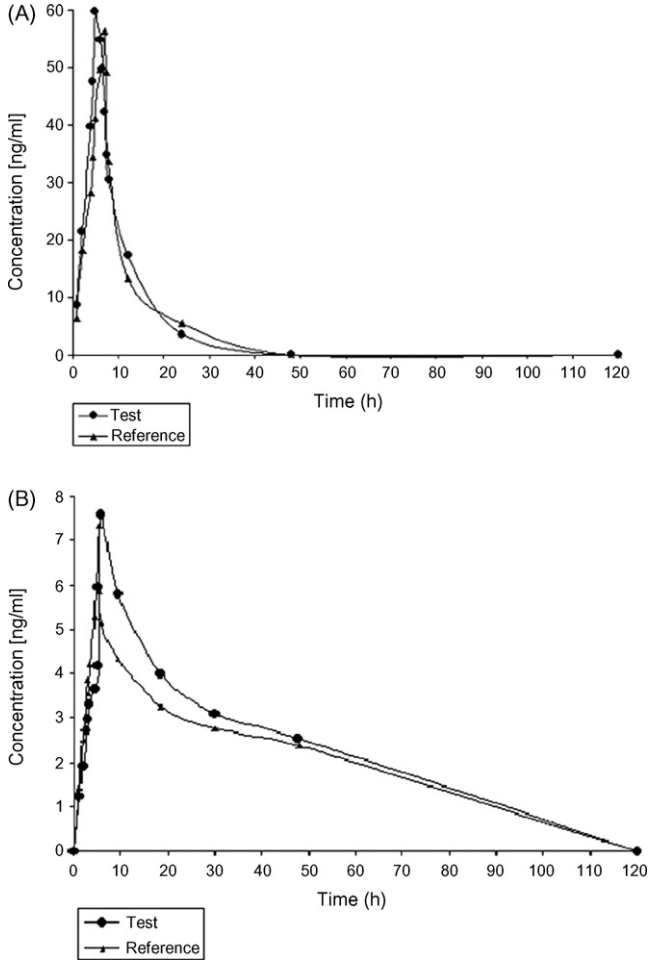


Fig. 4. Mean plasma concentration–time profiles for (A) MPS and (B) AM after oral administration of test preparation (FDC tablet containing MPS 50 mg ER and AM 5 mg) and reference preparation Met XL AM* 50 (FDC tablet containing MPS 50 mg ER and AM 5 mg) to Indian healthy human volunteers.

3.8. Application of LC–MS/MS method in pharmacokinetic study (pharmacokinetic variables study)

This method was successfully applied to the pharmacokinetic study of FDC of MPS and AM in human plasma. The mean (±S.D.) plasma concentration–time profile for a healthy human volunteer after a single oral administration of 50 mg of metoprolol succinate as ER and 5 mg of amlodipine besylate, is shown in Fig. 4. The values of the main pharmacokinetic parameters are shown in Table 6. Pharmacokinetic parameters (T_{max}, C_{max}, AUC_{0-t}, $AUC_{0-\infty}$, $t_{1/2}$ and K_{el}) were similar between the reference and test products.

4. Conclusion

The method described is highly specific due to the inherent selectivity of tandem mass spectrometry. The method demonstrates high throughput capability because of the short time required for analysis. Both the analytes were found to be stable in human plasma for 30 days when stored at −20 °C. A simple and convenient extraction procedure makes this method more feasible for the bio analysis of MPS and AM. It is expected that this method can be applied to clinical and toxicological studies. This method can also be applied to study the pharmacokinetic parameters of MPS and AM in healthy human volunteer. This study may be extended to determine the pharmacokinetics of MPS and AM in patients with specific illness and also to examine the drug-drug or drug-food interaction in combination therapy.

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