Bioanalytical method development and validation for determination of metoprolol tartarate and hydrochlorothiazide using HPTLC in human plasma

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A simple, sensitive, rapid and economic chromatographic method has been developed for determination of metoprolol tartarate and hydrochlorothiazide in human plasma using paracetamol as an internal standard. The analytical technique used for method development was high-performance thin-layer chromatography. HPTLC Camag with precoated silica gel Plate $60F_{254}$ (20 cm×10 cm) at 250 µm thicknesses (E. Merck, Darmstadt, Germany) was used as the stationary phase. The mobile phase used consisted of chloroform: methanol: ammonia (9:1:0.5v/v/v). Densitometric analysis was carried out at a wavelength of 239 nm. The rf values for hydrochlorothiazide, paracetamol and metoprolol tartarate were 0.13±0.04, 0.28±0.05, 0.48±0.04, respectively. Plasma samples were extracted by protein precipitation with methanol. Concentration ranges of 200, 400, 600, 800, 1000, 1200 ng/mL and 2000, 4000, 6000, 8000, 10000, 12000 ng/mL of hydrochlorothiazide and metoprolol tartarate, respectively, were used with plasma for the calibration curves. The percent recovery of metoprolol tartarate and hydrochlorothiazide was found to be 77.30 and 77.02 %, respectively. The stability of metoprolol tartarate and hydrochlorothiazide in plasma were confirmed during three freeze-thaw cycles (-20 °C) on a bench for 24 hours and post-preparatively for 48 hours. The proposed method was validated statistically and proved suitable for determination of metoprolol tartarate and hydrochlorothiazide in human plasma.

Uniterms: High performance thin layer chromatography/quantitative analysis. Metoprolol tartarate/determination in plasma. Hydrochlorothiazide/determination in plasma. Human plasma/quantitative analysis. Protein precipitation.

Um método simples, sensível, rápido e econômico empregando a cromatografia em camada delgada de alta eficiência (HPTLC) foi desenvolvido para determinação do tartarato de metoprolol e hidroclorotiazida em plasma humano, usando paracetamol como padrão interno. Placas prontas de sílica-gel $60F_{254}$ (20 cm×10 cm), 250 µm de espessura, para HPTLC Camag (E. A Merck, Darmstadt, Alemanha) foramutilizadas como fase estacionária. A fase móvel utilizada consistiu de clorofórmio: metanol: amônia (9:1:0,5 v/v/v). A análise densitométrica foi realizada no comprimento de onda 239 nm. Os valores de Rf de hidroclorotiazida, paracetamol e tartarato de metoprolol foram 0,13±0,04, 0,28±0,05, 0,48±0,04 respectivamente. As proteínas do plasma foram extraídas por precipitação com metanol. Para construção das curvas de calibração, empregaram-se intervalos de concentração de 200, 400, 600, 800, 1000, 1200 ng/mL e 2000, 4000, 6000, 8000, 10000, 12000 ng/mL de hidroclorotiazida e tartarato de metoprolol, respectivamente. Os percentuais de recuperação do tartarato de metoprolol e de hidroclorotiazida foram de 77,30 e 77,02, respectivamente. A estabilidade do tartarato de metoprolol e de hidroclorotiazida no plasma foi confirmada durante três ciclos de congelamento e descongelamento (-20 °C), durante 24 horas e póspreparação durante 48 horas. O método proposto foi validado estatisticamente, sendo adequado para determinação do tartarato de metoprolol e hidroclorotiazida em plasma humano.

Unitermos: Cromatografia em camada delgada de alta eficiência/análise quantitativa. Tartarato de metoprolol/determinação em plasma. Hidroclorotiazida/determinação em plasma. Plasma humano/análise quantitativa. Precipitação de proteínas.

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INTRODUCTION

Chemically, hydrochlorothiazide (Figure 1) is 6-chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulphonamide-1,1-dioxide and metoprolol tartarate is 1-[4-(2-methoxyethyl)phenoxy]-3-(propan-2-ylamino) propane-2-ol. Hydrochlorothiazide (HCTZ) is one of the oldest thiazide diuretics used to treat hypertension. It is often prescribed in combination with other antihypertensive drugs such as β-blockers, angiotensin converting enzyme inhibitors, or angiotensin II receptor blockers (Wellington, 2002). A combination dosage form of HCT with the β1-selective (cardio selective) –β-blocker Metoprolol tartarate (MET) (Smilide, 1983), is more effective than either drug alone in the management of hypertension and is effective in patients unresponsive to immunotherapy with either agent (Papademetriou *et al.*, 2006; Hainer, 2007). For the determination of MET in human plasma, several methods based on high performance liquid chromatography (HPLC) with UV (Aqil *et al.*, 2007) and fluorescence (Zhaing, 2006) detection, gas chromatography-mass spectrometry (Yilmaz, 2009) and LC–MS/MS (Gowda *et al.*, 2007; Gao *et al.*, 2010) have been reported. Similarly, a method for the determination of HCT in human plasma has been developed based on HPLC with UV or diode array detection (Zendelovska, 2004; Erk, 2003) and LC–MS/MS (Ramakrishna *et al.*, 2005; Liu *et al.*, 2007). No HPTLC method has been developed for determination of metoprolol tartarate and hydrochlorothiazide in human plasma by protein precipitation. Therefore, we developed a new simple, sensitive, rapid and economic high-performance thin-layer chromatographic method for determination of metoprolol tartarate and hydrochlorothiazide in human plasma by protein precipitation using paracetamol as an internal standard.

We preferred HPTLC over HPLC because the former method is useful for simultaneous processing of sample and standard, allowing better analytical precision and accuracy, several analysts to work simultaneously, and shorter analysis time as well as offering lower cost per analysis, low maintenance cost, simple sample preparation, handling of samples of divergent nature, no prior treatment for solvents such as filtration and degassing, low mobile phase consumption per sample, no interference from previous analysis, fresh stationary and mobile phases for each analysis, no contamination, visual detection, an open system, on UV-absorbing compounds detected by post-chromatographic derivatization.

MATERIAL AND METHODS

Instrumentation

HPTLC Camag with precoated silica gel Plate 60F254 (20 cm×10 cm) at 250 μm thicknesses (E. Merck, Darmstadt, Germany) was used as the stationary phase. Sample application was done by using a Camag 100 μL syringe and Camag Linomat V applicator. The sample was sprayed in the form of narrow bands of 8 mm length at a constant rate of 2 μL/s. Linear ascending development was carried out in a 20cm×10cm twin trough glass chamber (Camag, Muttenz, Switzerland). The densitometric scanning was performed by using Camag TLC scanner III
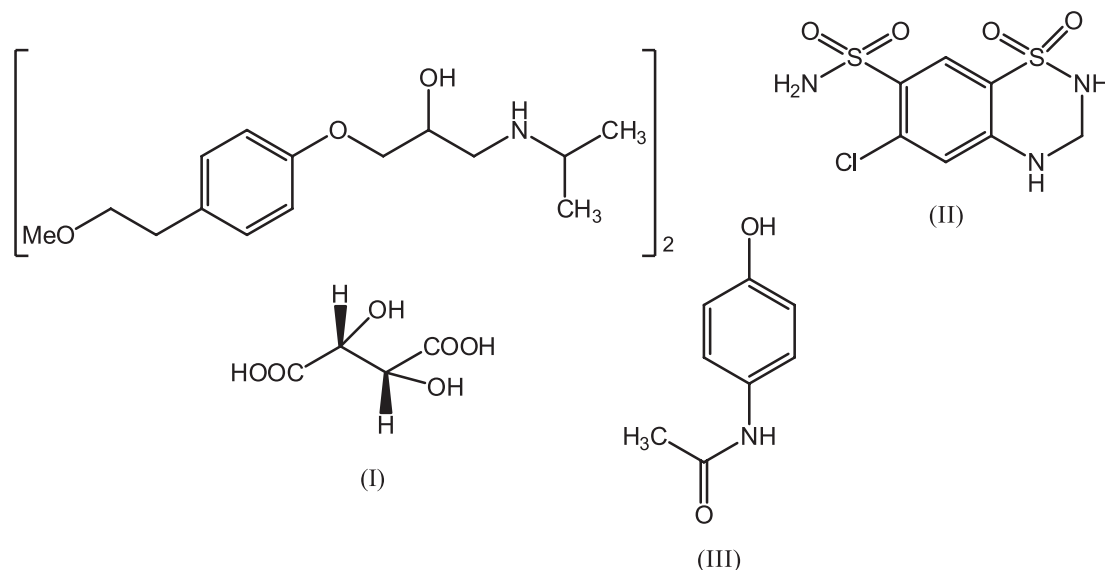
FIGURA 1 - Structure of metoprolol tartarate (I), hydrochlorothiazide (II) and paracetamol (IS) (III).

supported by win CATS software (V 1.4.2.8121 Camag). Evaluation of the chromatogram was done by using peak areas.

Chemicals

Metoprolol tartarate (Ajanta Pharmaceuticals, Mumbai, Maharashtra, India), hydrochlorothiazide (Elder pharmaceuticals, Mumbai, Maharashtra, India) and paracetamol (Kirti Pharmachem Ltd., Sinner, Maharashtra, India) had 99.80%, 98.70% and 100.1% purity, respectively. These were used as received without checking their purity. The HPLC grade methanol and Analytical Reagent grade Chloroform were purchased from s d Fine Chem. Ltd., Mumbai, India. The human plasma used for the research work was supplied by Arpan Blood Bank, Nashik, Maharashtra, India.

Preparation of stock solution and working standard solution

Stock solutions of 1.0 mg/mL each of metoprolol tartarate, hydrochlorothiazide and paracetamol were prepared in methanol.

Preparation of plasma sample

For HPTLC, in a 15mL centrifuge tube, 2, 4, 6, 8, 10, 12 µL of working stock solution of hydrochlorothiazide was added to drug-free plasma to provide calibration standards of 200, 400, 600, 800, 1000, 1200 ng/mL while 20,40,60,80,100,120 µL of working stock solution of metoprolol tartarate was added to drug-free plasma to provide calibration standards of 2000, 4000, 6000, 8000, 10000, 12000 ng/mL and 1000 ng/mL of paracetamol (internal standard) and kept constant. The quality control (QC) samples were prepared in plasma in the concentration range of 400, 800, and 1200 ng/mL and 4000, 8000, and 12000 ng/mL. Protein precipitation and extraction was carried out by using methanol 3 mL and acetonitrile 0.1 mL by vigorous vortex mixing using a Remi mixer for 1 min and centrifuging at 5,000 rpm for 10 min. The organic phase was recovered and evaporated to dryness on a hot plate. The residual mass was reconstituted with 1 mL methanol. The analysis was carried using HPTLC.

Chromatographic conditions

Mobile phase was selected as a mixture of chloroform, methanol and ammonia in the ratio of (9.0:1.0:0.5, v/v/v) for the development of plates. Time

for chamber saturation was optimized to 30 min. The length of chromatographic development was 70 mm. The densitometric scanning was performed at 239 nm.

Method validation

The method was validated for sensitivity, selectivity, precision, accuracy, linearity, recovery and stability. The validation of the method was based on FDA guidelines and on standard Bioanalytical method validation recommendations. The selectivity of the method was investigated by analyzing six blank plasma samples. Each blank sample was tested for interference using the proposed extraction procedure. Five replicates of three QC samples (low, mid and high) were used for the determination of precision and accuracy. Intra-day and inter-day precision were carried out. Precision and accuracies showed 15% relative standard deviation (RSD) from nominal values and at LLOQ these were both 20%. The recovery of metoprolol tartarate and hydrochlorothiazide was calculated by comparing the peak areas of low, mid, and high quality control sample (400, 800, 1200 ng/mL and 4000, 8000. 12000 ng/mL, respectively) prepared in plasma (extracted) with unextracted metoprolol tartarate and hydrochlorothiazide, respectively.

Stability experiments were undertaken to detect degradation of Metoprolol tartarate and hydrochlorothiazide under specific conditions. Freeze–thaw stability was determined at two QC concentrations (low, high) after freezing (−20 °C) and thawing for three cycles and compared with the nominal value. Bench-top stability was assessed for low and high QC samples by comparing with the nominal value which was stored at room temperature for 12 h. The effect of storage within the auto-sampler was assessed by comparing QC samples injected immediately after preparation with those left in the auto-sampler for 48 h.

RESULT AND DISCUSSION

Extraction procedure optimization

One of the most difficult parts during the method development was to achieve high and reproducible recovery from the solvent used for extraction of the drug. Different solvents were tried for the extraction of metoprolol tartarate and hydrochlorothiazide from human plasma. First, 3 mL each of chloroform and toluene were tried for the precipitation of plasma but the recovery proved very low. It yielded 55-75% recovery because of less precipitation of protein from plasma. Finally,

methanol was tried and 70–80% recovery obtained. It was found that the addition of acetonitrile (0.1 mL) increased the precipitation of protein and also the recovery which was reproducible and high as compared to other solvents. Thus, methanol and acetonitrile (3.0 mL: 0.1 mL) was kept as the final solvent for extraction of hydrochlorothiazide and metoprolol tartarate. Recovery was done in plain extracting solvent from human plasma.

Optimization of chromatographic conditions

Initially, plain solvents such as methanol, ethyl acetate, chloroform, toluene, acetone were tried. The spots were developed with chloroform and methanol but no proper resolution observed among hydrochlorothiazide, metoprolol tartarate and paracetamol, also showing the tailing. Subsequently, chloroform and methanol at the ratio of (8:2, v/v) was tried but again no proper resolution was obtained. The proportion of chloroform was then increased and of methanol decreased by 1mL, showing good resolution. Lastly, the addition of Ammonia solution at 0.5 mL helped to reduce the tailing effect and good resolution with symmetrical peaks of hydrochlorothiazide, paracetamol and metoprolol tartarate were obtained. Finally, the mobile phase used consisted of chloroform: methanol: Ammonia (9:1: 0.5, v/v/v) which allowed good resolution of peaks for hydrochlorothiazide, metoprolol tartarate and paracetamol. The R_f values for hydrochlorothiazide, paracetamol and metoprolol tartarate were found to be 0.13±0.04, 0.28±0.05 and 0.48±0.04, respectively. Well-defined spots were obtained

by prewashing the plate using methanol followed by activating at 120 °C for 20 min. The chamber was saturated with mobile phase for 25 min at room temperature, yielding reproducible R_f values for hydrochlorothiazide, paracetamol and metoprolol tartarate, respectively (Figure .2).

Calibration curves

A six-point calibration curve was constructed by plotting the peak response ratio of hydrochlorothiazide to paracetamol versus concentration of hydrochlorothiazide and metoprolol tartarate to paracetamol versus concentration of metoprolol tartarate in plasma. Correlation coefficients were 0.9979 and 0.9954 for hydrochlorothiazide and metoprolol tartarate, respectively. Linearities were found over the range 2-12 µL/mL and 20-120 µL/mL for hydrochlorothiazide and metoprolol tartarate, respectively. The lower limit of quantification was defined as the lowest concentration in the calibration curve. The hydrochlorothiazide and metoprolol tartarate can be determined at LLOQ 2 µL/mL and 20 µL/mL.

Recovery

Absolute recovery was calculated by comparing peak areas obtained from freshly prepared sample extracted with unextracted standard solutions of the same concentration. Recovery data was determined in triplicates at three concentrations (low, mid, high) as recommended by the FDA guidelines (US Department, 2001). The
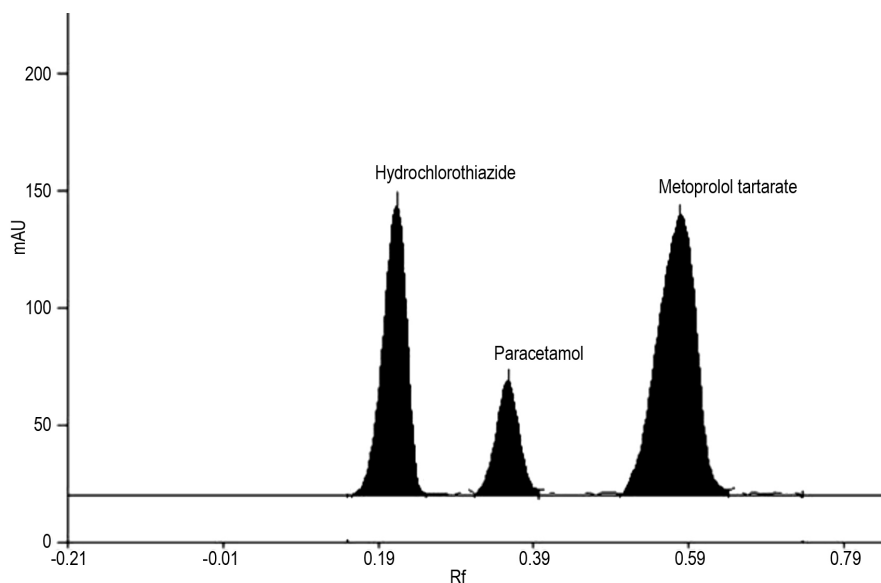
FIGURE 2 - Densitogram of hydrochlorothiazide (Rf(0.13±0.04), paracetamol Rf(0.28±0.05) and metoprolol tartarate Rf(0.48±0.04).

recoveries of hydrochlorothiazide and metoprolol tartarate for HPTLC recovery at the three concentrations 400, 800, 1200 ng/mL and 4000, 8000, 12000 ng/mL were found to be 77.33, 74.65, 79.94% and 70.59, 84.91, 75.57 %, respectively (Table I).

TABLE I - Result of recovery of hydrochlorothiazide and metoprolol tartarate in human plasma

Sr. No.	Concentration		Recovery		%RSD	
	HCTZ	MET	HCTZ	MET	HCTZ	MET
1	400	4000	77.33	70.59	7.92	11.2
2	800	8000	74.65	84.91	7.32	4.5
3	1200	12000	79.94	75.57	4.9	3.8

Precision and accuracy

Precision of the method was determined by repeatability (intraday) and intermediate precision (inter-day) and accuracy for a set of quality control (QC) samples (low, mid, high) in replicate (n = 5). The inter-day and intra-day precision and accuracy for the hydrochlorothiazide and metoprolol tartarate evaluated by assaying the QC samples (low, mid, high) (n = 5) were expressed in (%RSD). In this assay, the intra-run precision was found to be in the range of 0.53-3.17% and 1.29-4.24% whereas the inter-run precision was 1.2-1.51% and 1.43-1.7%. The accuracy was within 1.10–2.67%. The above values were within the acceptable range, showing that the methods are accurate and precise. The low percent relative standard deviation (%RSD) and percent relative error (%RE) were within acceptable limits. The results of inter-day, intra-day precision, and accuracy for the hydrochlorothiazide and metoprolol tartarate are shown in Table II.

Sensitivity and selectivity

Selectivity or specificity should be assessed to show that the intended analytes are measured and that their quantitation is not affected by the presence of the biological matrix. For the HPTLC method by protein precipitation, as shown in Figure 3, there was no interference by the biological matrix in the quantitation of hydrochlorothiazide and metoprolol tartarate. There were no changes in the retention factor of hydrochlorothiazide and metoprolol tartarate, where the method proved selective. Sensitivity of the method is defined as the lowest concentration that can be measured with an acceptable limit of accuracy and precision which is lower than 20% (Bansal, 2007). The accuracy and precision at the lower limit of quantitation (LLOQ) was analyzed by using five replicates (n = 5) of the sample at the LLOQ concentration. The accuracy was determined by %RE at this LLOQ concentration. The lower limits of quantitation detected were found to be 200 ng/mL and 2000 ng/mL with %RE = 17.8, 13.43 and %RSD= 10.74, 4.11 for hydrochlorothiazide and metoprolol tartarate, lying within the acceptable limit.

Stability

In bench-top stability, the low and high QC sample were thawed and left at room temperature for 12 h. Comparison of the results for the QC sample (low and high) with freshly prepared stock solution showed that there was no significant difference between response of freshly prepared solution and sample of hydrochlorothiazide and metoprolol tartarate after 12 h. Freeze–thaw stability was determined after two freeze–thaw cycles for three replicates of low and high QC samples. The samples were stored at −20 ◦C temperature for 24 h. They were then thawed at room temperature. No significant difference between freeze–thaw samples and freshly prepared samples was observed. The result of stability experiments showed that no significant degradation occurred at ambient temperature for 48 h for post-preparative stability. Results of stability for the HPTLC method are shown in Table III.

TABLE II - Results of precision of hydrochlorothiazide and metoprolol tartarate in human plasma

Precision	Concentration (ng/mL)		%RSD		%RE	
	HCTZ	MET	HCTZ	MET	HCTZ	MET
	400	4000	2.09	1.20	6.37	4.55
INTRA	800	8000	0.55	0.92	14.69	10.01
	1200	12000	1.57	1.24	7.10	9.76
	400	4000	4.80	2.43	6.88	11.44
INTER	800	8000	2.44	7.14	15.82	12.53
	1200	12000	1.12	0.73	6.69	8.43

TABLE III - Stability study of metoprolol tartarate and hydrochlorothiazide in human plasma

Parameters	Concentration(ng/mL)		S.D		%RE	
	HCTZ	MET	HCTZ	MET	HCTZ	MET
Freeze-thaw stability						
Low	200	2000	16.96	55.73	7.14	14.08
High	1200	12000	44.60	301.67	10.36	9.01
Bench-top stability						
Low	200	2000	4.86	81.95	5.23	12.76
High	1200	12000	60.20	439.23	7.30	15.25
Post-preparative stability						
Low	200	2000	2.12	33.24	3.18	9.03
High	1200	12000	16.69	80.18	4.69	9.91

CONCLUSION

The proposed HPTLC method for the estimation of metoprolol tartarate and hydrochlorothiazide in human plasma by protein precipitation is both selective and sensitive. The sensitivity of the method is suitable for handling various plasma levels of the drug. The method is economical and faster than previously published methods.

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