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Development and validation of a liquid chromatography/tandem mass spectrometry assay for the simultaneous determination of nateglinide, cilostazol and its active metabolite 3,4-dehydro-cilostazol in Wistar rat plasma and its application to pharmacokinetic study

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Abstract

Nateglinide (NTG), an insulin secretogogue, has been studied in rats for drug–drug interaction with cilostazol (CLZ), an antiplatelet agent commonly used in diabetics. We developed a liquid chromatography tandem mass spectrometry (LC–MS/MS) based method that is capable of simultaneous monitoring plasma levels of nateglinide, cilostazol, and its active metabolite 3,4-dehydro-cilostazol (DCLZ). All analytes including the internal standard (Repaglinide) were chromatographed on reverse phase C_{18} column (50 mm × 4.6 mm i.d., 5 μm) using acetonitrile: 2 mM ammonium acetate buffer, pH 3.4 (90:10, v/v) as mobile phase at a flow rate 0.4 ml/min in an isocratic mode. The detection of analyte was performed on LC–MS/MS system in the multiple reaction monitoring (MRM) mode. The quantitations for analytes were based on relative concentration. The method was validated over the concentration range of 20–2000 ng/ml and the lower limit of quantitation was 20 ng/ml. The recoveries from spiked control samples were >79% for all analytes and internal standard. Intra- and inter-day accuracy and precision of validated method were with in the acceptable limits of <15% at all concentration. The quantitation method was successfully applied for simultaneous estimation of NTG, CLZ and DCLZ in a pharmacokinetic drug–drug interaction study in Wistar rats.
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1. Introduction

Therapeutic drug management of antidiabetic agents (ADA) and antiplatelet drugs is an aid in the clinical management of diabetic patients with peripheral arterial diseases (PAD). Monitoring these drugs is desirable for adjusting doses, to provide safe and efficacious individualized dosage regimens, avoiding side effects and assessing patient compliance.

Among ADAs, nateglinide (NTG), a D-phenylalanine derivative, a potent insulin secretogogue designed to restore the early phase insulin secretion [1]. It increases pancreatic insulin secretion by competitively binding to sulphonylurea receptors (SUR), there by inhibiting KATP channels and thus reduces blood glucose levels in diabetic patients [2,3]. It has rapid onset and short duration of insulinotropic effects [4,5]. It is metabolized predominantly by CYP2C9 to its hydroxy and glucuronide metabolite [6].

The drug commonly used for antiplatelet therapy in diabetics is cilostazol (CLZ) along with ADAs. Cilostazol, a selective PDE-III enzyme inhibitor, with antithrombotic, vasodilator, antimitogenic and cardiotonic properties used for the treatment of intermittent claudication in PAD [7,8]. In vitro metabolism studies in human liver microsomes revealed that hepatic cytochrome P450 including CYP3A4 and CYP2C19 are involved in the metabolism of cilostazol [9]. The major active in vivo metabolites found in rat after administration of cilostazol was 3,4-dehydro cilostazol (DCLZ) and the minor was 4′-trans-hydroxy-cilostazol along with the other metabolites [10].

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The drug–drug interaction could be possible since prescriptions in diabetics are commonly prescribed with antiplatelet agents along with ADAs. There are more prescriptions of using NTG and CLZ in combination in diabetics and no such information on the drug–drug interaction effect of these two drugs when given together. While investigating the analytical methods in this regard, we found several individual methods for estimating these drugs alone in biological fluids by reverse phase chromatographic methods [11–20]. All these individual methods suffer from long analysis time, low sensitivity and difficult extraction procedures. These delay the analysis time and also drastically affect column life and consume a lot of reagents. To our knowledge, no prior reports have described a LC–MS/MS-based method for simultaneous determining NTG, CLZ and DCLZ from plasma. Hence, we developed a reverse phase HPLC method for simultaneous estimation of NTG, CLZ and DCLZ on C_{18} column using tandem mass spectroscopy detection and validated before using in our preclinical experiments. The present study describes a rapid, specific and simple liquid–liquid extraction method using liquid chromatography tandem mass spectrometry (LC–MS/MS) for the simultaneous determination of NTG and CLZ along with its major active metabolite DCLZ in rat plasma suitable for pharmacokinetic and drug–drug interaction studies. The method was validated using authentic pure standards. This method was successfully applied in the pharmacokinetic drug–drug interaction study of NTG with CLZ in rats after oral administration in combination.

2. Experimental

2.1. Reagents and standards

Pure reference standards of cilostazol, 3,4-dehydrocilostazol, nateglinide and repaglinide (IS) were obtained from Glenmark Pharmaceuticals Limited (Mumbai, India). Acetonitrile (HPLC grade), methyl *t*-butyl ether (TBME) and methanol (HPLC grade) were purchased from Thomas Baker, India. Ammonium acetate (GR-grade) was procured from E Merck (India) Ltd., India. Formic acid was obtained from Sigma Aldrich, Germany. Ultra pure water of 18 MΩ/cm was obtained from Milli-Q PLUS purification system (Millipore, USA). Blank rat plasma was collected from healthy, drug free Wistar rats at the Glenmark research unit, Navi Mumbai (India). Plasma was obtained by centrifuging the K_2-EDTA (di-potassium ethylene diamine tetra acetic acid, Sigma Aldrich, Germany) blood at 3000 rpm for 10 min.

For pharmacokinetic studies, young and healthy male Wistar rats weighing 200 ± 30 g were inbred in animal house facility of Glenmark Pharmaceuticals Ltd., at R&D Centre, MIDC Mahape, Navi Mumbai and housed in appropriate stainless steel cages in standard laboratory conditions with regular 12 h day–night cycle in well-ventilated room with an average temperature of 25–28 °C and relative humidity of 40–60%. Standard pelleted laboratory chow (Goldmohar Laboratory Animal Feeds, Chandigarh, India) and water allowed *ad libitum* to rats. All ethical guidelines for maintenance and experimental studies with Wistar rats were followed.

2.2. Stock solution, calibration standards and quality control samples

Standard stock solutions of NTG, CLZ, DCLZ and IS were prepared in methanol with a final concentration of 1 mg/ml. These solutions were stored at 2–8 °C until use. The IS stock solution was diluted to achieve a final concentration of 2.5 μg/ml with the diluent (acetonitrile:water, 70:30, v/v) solution. Analytical standards for NTG, CLZ and DCLZ were prepared in acetonitrile:water (70:30, v/v) over a concentration range of 21.1–2036.8 ng/ml, 21.7–2090.0 ng/ml and 21.8–2100.0 ng/ml, respectively by serial dilution, and same concentration range for calibration curve were prepared in blank normal rat plasma. Quality control (QC) samples at four different concentration levels (21.1, 45.6, 817.3, 1629.4 ng/ml for NTG, 21.7, 47.2, 838.7, 1672.0 ng/ml for CLZ and 21.8, 47.4, 842.7, 1680.0 ng/ml for DCLZ as low 1, low 2, medium and high, respectively) were prepared in three sets independent of the calibration standards. During analysis, these QC samples were spaced after every six to seven unknown samples.

2.3. Sample preparation

Sample preparation involved liquid–liquid extraction (LLE) method using of 0.1N HCl (50 μl) and TBME (1.5 ml). The processing volume of plasma was fixed as 50 μl. The IS solution was spiked in to such aliquots as to give a final concentration of 62.5 ng/ml of repaglinide and vortex mixed (Type 37600 mixer, Thermolyne, USA) prior to the addition of the extraction solvent. After mixing thoroughly with vortex mixer for 10 min and centrifugation (Megafuge 3SR, Heraeus, Germany) at 10,000 rpm for 5 min, the supernatant collected was dried under nitrogen stream (Nitrogen Evaporator, Zymark, USA). The residue was reconstituted in 100 μl of mobile phase and 5 μl was injected onto the LC–MS/MS system. For determination of matrix effect, control drug free plasma was extracted using TBME as described above and final supernatant was evaporated to dryness. Dry extracts were dissolved using analytes and IS standard solutions at limit of quantification (LOQ) concentration level that represent 100% recovery. Matrix effect was determined by comparing the analytical response of these samples with that of standard solutions.

2.4. Chromatographic condition

A PerkinElmer Series 200 pump (Norwalk, CT, USA) consisting of flow control valve, vacuum degasser (Series 200 vacuum degasser) operated in isocratic mode to deliver the mobile phase at flow rate of 0.4 ml/min. The chromatographic system consisted of reverse phase C_{18} column (50 mm × 4.6 mm i.d., 5 μm) (Hypurity®, Thermo Electron, USA) and mobile phase consists of 90% (v/v) solvent A: acetonitrile and 10% (v/v) solvent B: ammonium acetate buffer, 2 mM (pH ~ 3.4 adjusted with 0.1% formic acid). The samples (5 μl) were injected on to the LC–MS/MS system through an auto injector. The auto sampler temperature was kept at 10 °C and the column oven was maintained at 40 °C.

2.5. Mass spectrometric condition

Mass spectrometric detection was performed on API 3200–QTRAP mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada) equipped with a Turbo V IonSpray ionization source operating in ESI positive ion mode. In mass spectrometer, zero air was used as nebulizing gas (GS 1) and turbo gas (GS 2) while nitrogen as curtain gas (CUR) and collision activated dissociation (CAD) gas and these gases were constantly supplied from a gas generator (Peak Scientific, USA). MS and MS/MS condition for pure standards of NTG, CLZ, DCLZ and IS were optimized by continuous infusion at 5 μl/min using syringe pump (Model '11', Harvard apparatus, Inc., Holliston, MA, USA). MS/MS analyses of all analytes were performed using nitrogen as CAD gas. The most abundant product ion of each component was selected to build multiple reaction monitoring (MRM) method. The transitions monitored were *m/z* 318 > 166.2, 370 > 288.3, 368.3 > 286.2, and 453.3 > 230.4 for components NTG, CLZ, DCLZ and IS, respectively.

Ion source gas (GS 1), ion source gas (GS 2), curtain gas (CUR), collision gas (CAD), ion spray voltage and temperature were set to 25, 35, 10, medium, 5500 kV and 400 °C, respectively. The mass spectrometer was operated in unit resolution for both Q1 and Q3 in the MRM mode, with a dwell time of 200 ms per MRM channel. All data were acquired within 3.5 min using Analyst 1.4.1 software (Applied Biosystems/MDS SCIEX).

2.6. Application to pharmacokinetic study

The method was successfully applied to generate the plasma concentration versus time profile of test drugs (NTG and CLZ) as well as to detect its metabolite (DCLZ) in plasma following simultaneous oral administration at 10 mg/kg doses of NTG and CLZ in male Wistar rats. Oral formulations were prepared in suspension form by triturating accurately weighed amount of powdered compound in methyl cellulose solution (0.5%, w/v water) in gravimetric dilution pattern. Oral doses of NTG and CLZ (10 mg/kg) were administered using an oral gavage at 5 ml/kg volume in rats after overnight fasting (12 h) and these animals were continued for fasting till 4 h post dose. The blood samples (0.15 ml) were collected from retro orbital sinus at predose, 10, 15, 30 min and 1, 2, 3, 4, 6, 8, 12 and 24 h post dose and were kept on ice bath till further processing. These samples were separated for plasma by centrifuging at 4 °C for 10 min at 3000 rpm and then stored at −70 °C till further analysis. These samples were analyzed for simultaneous estimation of the levels of NTG, CLZ and its active metabolite DCLZ.

3. Results and discussion

3.1. Mass spectrometry

In order to find most sensitive ionization mode for the components studied, ESI positive ion mode and ESI negative ion mode were tested with various combination of mobile phase, i.e., acetonitrile and water/ammonium acetate buffer (2 mM)/formic acid (0.1%) in positive and negative ionization mode. It was observed that the signal intensity for $[M+H]^+$ ions in ESI positive ion mode were 2–10-fold higher for all components in analyses using acetonitrile:ammonium acetate buffer (2 mM), versus experiments run with ESI negative ion mode. The protonated molecular ion of $[M+H]^+$, *m/z* 318, 37, 368.3 and 453.3 amu were obtained for NTG, CLZ, DCLZ and IS, respectively. No significant solvent adduct ions or fragment ions were observed in the full scan spectra of all the compounds. Thus, it was decided to utilize positive ion mode for detection and quantization of $[M+H]^+$ ions, which on fragmentation gave prominent and stable product ions. The optimized declustering potentials for the protonated $[M+H]^+$ of component NTG, CLZ, DCLZ and IS, were found to be 48, 42, 56 and 70 V, respectively.

3.2. Liquid chromatography

Acetonitrile rather than methanol was chosen as the organic modifier because of its better peak shape. Moderately high acidic ammonium acetate buffer 2 mM, pH ∼ 3.4, was required to achieve acceptable peak width and shapes. A reverse phase C_{18} column (50 mm × 4.6 mm i.d., 5 μm) (Hypurity®, Thermo Electron, USA) with acetonitrile:ammonium acetate buffer in gradient mode was applied in final LC method. Within the total analysis time of 3.5 min, all components were eluted in 1.8–2.3 min. A column with narrow internal diameter (Inertsil ODS-3, 100 mm × 4.6 mm i.d., particle size, 3 μm, GL sciences, Japan) was tested in the development stage with the solvent flow rate decreased to 0.3 ml/min. Despite the slightly improved resolution, retention was delayed for component NTG. Additionally, the analysis time expanded to 6 min.

3.3. Optimization of LC–MS/MS condition

Final MRM transitions were selected on the basis of signal to noise ratio (S/N) ratio with on-column injection analysis. GS 1, GS 2, CUR, CAD, ion spray voltage and temperature were set to 25, 35, 10, medium, 5500 kV and 400 °C, respectively. The transitions selected were *m/z* 318 > 166.2, 370 > 288.3, 368.3 > 286.2, and 453.3 > 230.4 for components NTG, CLZ, DCLZ and IS, respectively. The fragment ions selected for final MRM method are given in Fig. 1. The selection of repaglinide, a meglitinide derivative as IS was based on its structural similarity with NTG. Hence, repaglinide was expected to behave closely in terms of ionization giving better results for linearity and quantitation.

3.4. Sample clean up

The next step was to develop simple and efficient sample clean up devoid of matrix effect and interference from endogenous plasma components for estimation of the analytes in rat plasma. Hence the precipitation method was tried initially with acetonitrile (1.5 ml) and it has shown ion enhancement for all the analytes. Further LLE using ether and different combinations of hexane and ethyl acetate (90–10%, v/v), *n*-hexane and isopropyl alcohol (2–5%, v/v) was tried but none of these was

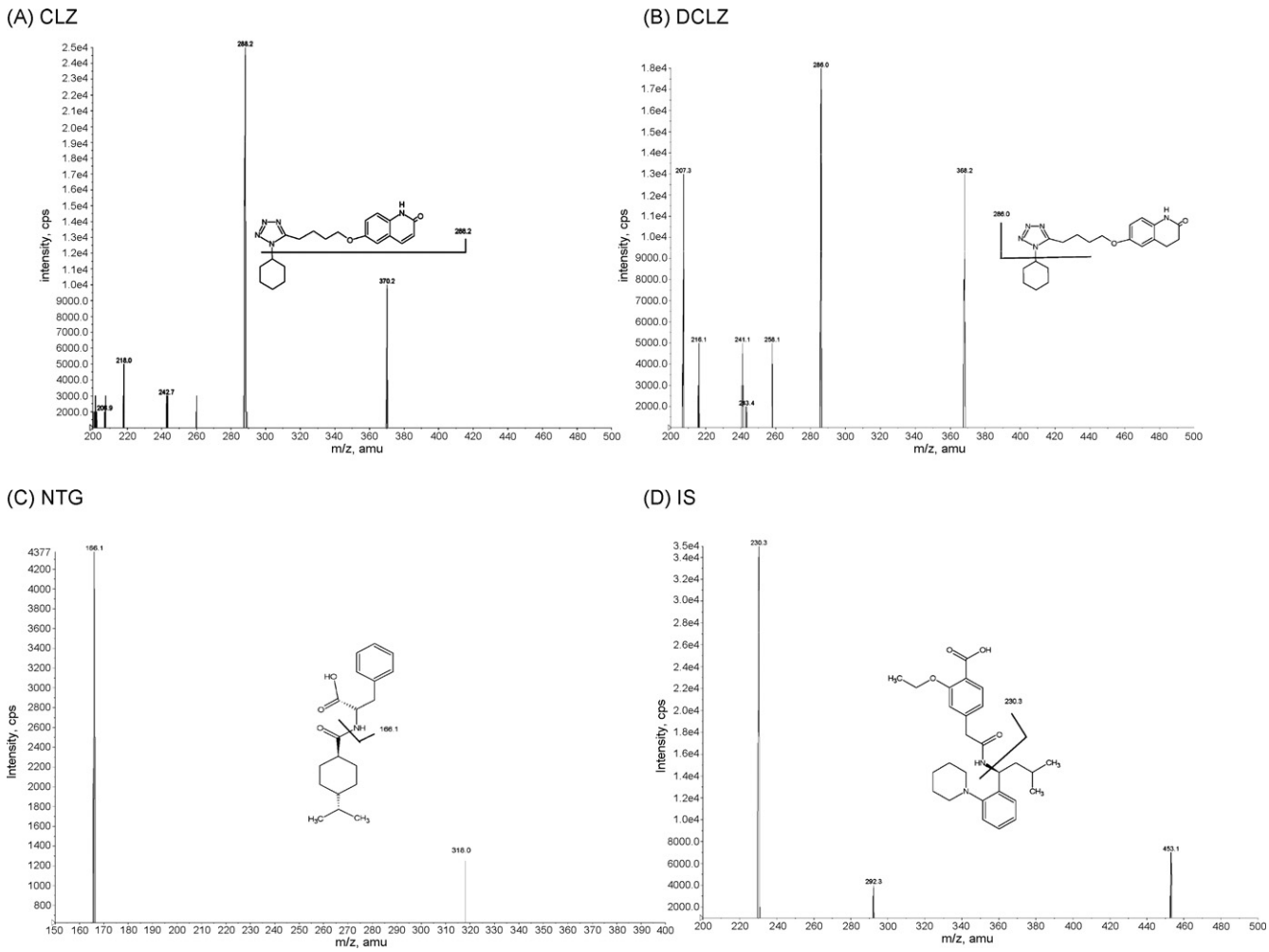

Fig. 1. Chemical structures and product ion spectra of $[M+H]^+$ of: (A) CLZ, (B) DCLZ, (C) NTG and (D) IS.

found suitable to give good and consistent recovery for all analytes. Finally, LLE using TBME was tried and found suitable to give optimum recovery for all analytes. The matrix effect was evaluated directly by extracting control drug free plasma and then spiking with the analyte at the LOQ concentration. There was no difference observed between the signal for the standard solution and the spiked extract at the LOQ concentration.

3.5. Method validation

Accuracy, precision, selectivity, sensitivity, linearity and stability were measured and used as the parameter to assess the assay performance. LC–MS/MS analysis of the blank plasma samples showed no interference with the quantification of components NTG, CLZ, DCLZ and the IS. The specificity of the method was established with pooled and individual plasma samples from eight different sources. Representative overlaid chromatogram of extracted blank rat plasma and blank plasma fortified with analytical standards and IS, demonstrating the specificity and selectivity of the method are shown in Fig. 2. The retention times of all the analytes and the IS showed less variability with a relative standard deviation (R.S.D.) well within acceptable limits of 5%.

3.5.1. Limit of detection (LOD) and LOQ

Two criteria were used to define LOQ: (1) the analytical response at LOQ must be five times the baseline noise and (2) the analytical response at LOQ can be detected with sufficient precision (15–20%) and accuracy (80–120%). LOD is defined as the lowest concentration of the analyte at which the signal is larger than three times the baseline noise. The measured LOQ and LOD values were 20 and 5 arbitrary units for all three analytes. These results well met the requirements of quantifications of all analytes in plasma.

3.5.2. Linearity

The peak area ratios of analytes to IS in rat plasma were linear over the concentration range 21.1–2036.8 ng/ml for NTG and 21.7–2090.0 ng/ml for CLZ and 21.2–2100.0 arbitrary unit for component DCLZ. The calibration model was selected based on the analysis of the data by linear regression with and without intercepts ($y = mx + c$ and $y = mx$) and weighting factors ($1/x$, $1/x^2$ and $1/\log x$). The best fit for the calibration curve could be achieved by a linear equation of $y = mx + c$ and a $1/x^2$ weighting factor for all components. The correlation coefficients (R) for all components were above 0.996 over the concentration range used. Table 1 shows the results obtained

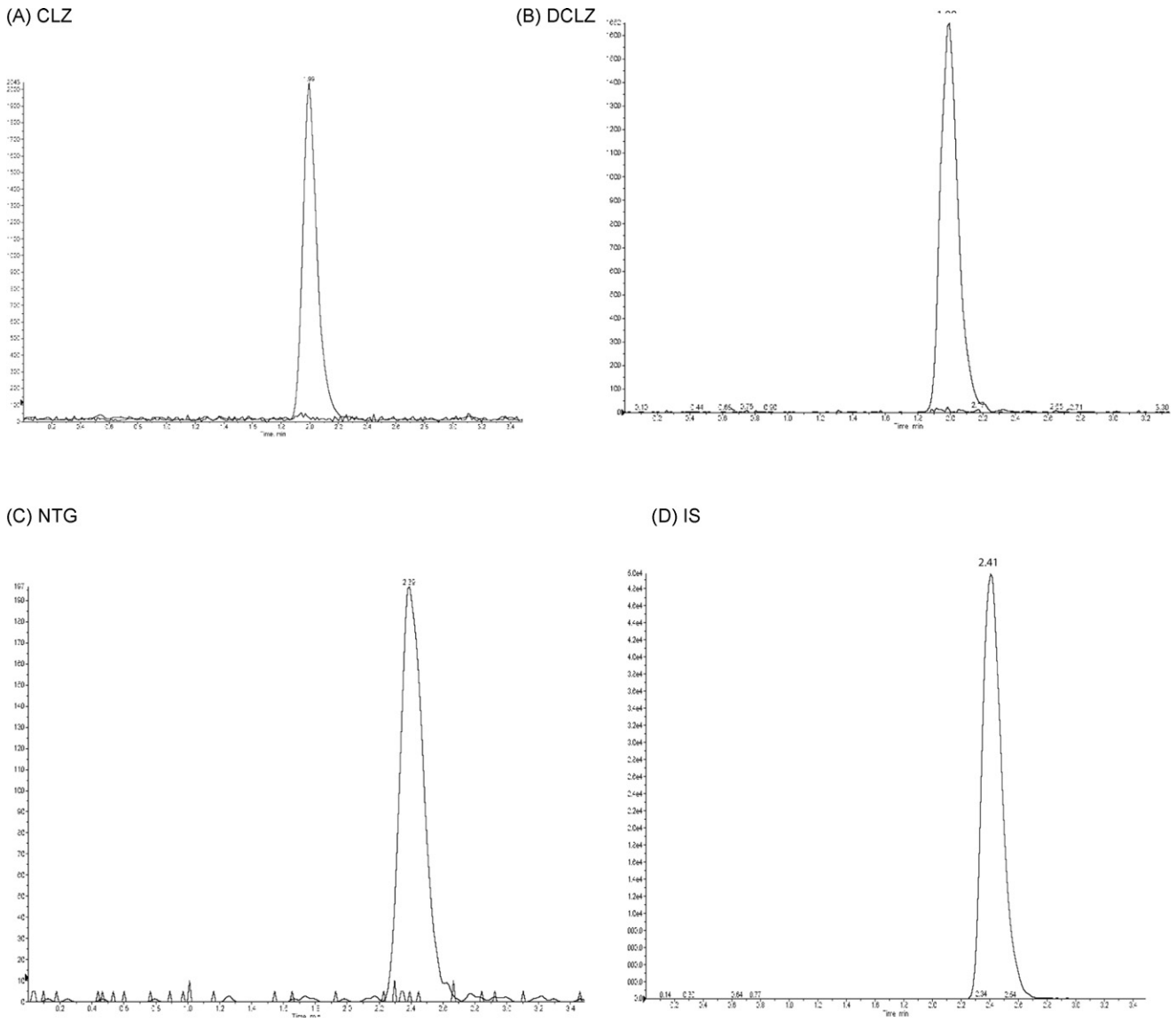

Fig. 2. Representative chromatograms (at respective LOQ) of: (A) CLZ, (B) DCLZ, (C) NTG and (D) IS in the fortified blank rat plasma overlaid with extracted blank rat plasma.

for summary parameters of linearities for CLZ, DCLZ and NTG.

3.5.3. Precision and accuracy

The intra-day precision (expressed by coefficient of variation of replicate analyses) was estimated on the three quality control levels and the inter-day precision on the nine calibration standard levels. Table 2 shows the results obtained for the intra-assay (variation intra-day) and inter-assay (variation inter-day) precision for NTG, CLZ and DCLZ. The precision for all these analytes under investigation were not exceeded 15% at any of the concentrations studied and well met the requirements of validation.

3.5.4. Recovery

The recovery of NTG, CLZ and DCLZ from plasma was estimated at their respective low, medium and high QC levels. Plasma samples (in six replicates) containing all analytes at QC concentration level were also spiked with IS at the working concentration of 62.5 ng/ml. The samples were subsequently processed using the procedure described previously. A second set of plasma samples was processed and spiked post-extraction with the same concentrations of the analytes and IS that actually existed in the pre-extraction spiked samples. Extraction recovery values for each analyte and IS were determined by calculating the ratios of the raw peak areas of the pre-extraction spiked samples to that of the samples spiked after extraction. The results are indicated in Table 3.

3.5.5. Stability

QC samples were subjected to short term and long term storage condition (−70 °C), freeze–thaw stability, auto-sampler stability and dry residue stability studies. All stability studies were carried out at two concentration levels (low and high QC) in six replicates.

Table 1
Summary parameters of four different linearities for CLZ, DCLZ and NTG

Drug	Linearity	Intercept	Slope	Correlation coefficient (r^2)
CLZ	1	0.00059	0.00122	0.9960
	2	0.00063	0.00105	0.9912
	3	0.00057	0.00149	0.9859
	4	0.00060	0.00122	0.9965
	Mean	0.00060	0.00125	0.9924
	S.D.	0.00002	0.00018	0.0050
	S.E.	0.00001	0.00009	0.0025
DCLZ	1	0.00089	−0.00488	0.9980
	2	0.00069	−0.00366	0.9930
	3	0.00065	−0.00317	0.9920
	4	0.00089	−0.00469	0.9989
	Mean	0.00078	−0.00410	0.9955
	S.D.	0.00013	0.00082	0.0035
	S.E.	0.00006	0.00041	0.0017
NTG	1	0.00011	−0.00079	0.9956
	2	0.00008	−0.00071	0.9950
	3	0.00010	−0.00055	0.9948
	4	0.00010	−0.00065	0.9967
	Mean	0.00010	−0.00068	0.9955
	S.D.	0.00001	0.00010	0.0009
	S.E.	0.00001	0.00005	0.0004

The bench top stability was studied for low and high QC samples kept at room temperature for 6 h freeze–thaw stability of low and high QC samples was evaluated after 3 freeze thaw cycles. The autosampler stability was studied for low and high QC samples kept at autosampler kept at 10 °C for 24 h. The freezer storage stability of the drug in plasma was determined by comparing the low and high QC samples stored for 30 days at −70 °C. The percentage stability was estimated by comparing the mean of back-calculated concentration of all analytes from the stored stability samples with that of freshly spiked QC samples. The results indicated that each analyte had an acceptable stability under those conditions, as shown in Table 4.

Table 3
Extraction recovery in rat plasma ($n = 6$)

Drug	Concentration (ng/ml)	Recovery (%)	R.S.D
CLZ	47.2	83.2	4.4
	838.7	91.0	5.3
	1672.0	85.9	6.9
DCLZ	47.4	82.5	3.1
	842.7	81.2	4.1
	1680.0	88.1	7.5
NTG	45.6	79.5	8.1
	817.3	86.5	7.1
	1629.4	87.6	5.0

3.5.6. Sample dilution

To demonstrate the ability to dilute and analyze samples containing all analytes at concentration above the assay upper limit of quantification, a set of plasma samples was prepared containing NTG, CLZ and DCLZ at a concentration of 6110, 6270 and 6300 ng/ml, respectively, and placed in a −70 °C freezer overnight prior to analysis. After thawing, certain aliquot was diluted either with 2 and 4 or 4 and 8 times Wistar rat plasma and analysed, respectively. The results of this experiment indicated that the dilution integrity of all the plasma samples was found to be less than 15% of their respective nominal concentrations.

3.5.7. Comparison of methods

Previous methods describing the determination of cilostazol and its metabolites involved liquid–liquid partitioning followed by solid phase extraction (SPE) using LC–MS/MS [11]. Other methods using HPLC-UV were involved with various gradient and isocratic conditions comprised of multiple steps of LLE or SPE procedures for estimating CLZ and its metabolites [10,12–15]. Most of the available methods for determination of NTG were involved using UV detector [16–19]. However, the reported method involves complex step extraction procedures and larger run times. Another method using MS detector [20] is being published recently and it described the multiple analyte

Table 2
Summary of precision and accuracy from QC samples in Wistar rat plasma

Drug	Spiked concentration (ng/ml)	Intra-day ($n = 6$)			Inter-day ($n = 4$)		
		Measured concentration (ng/ml) (mean ± S.D.)	R.S.D.	Nominal (%)	Measured concentration (ng/ml) (mean ± S.D.)	R.S.D.	Nominal (%)
CLZ	21.7	21.4 ± 2.6	12.2	98.7	21.2 ± 1.9	8.9	97.9
	47..2	47.5 ± 6.6	6.3	100.7	47.3 ± 5.5	11.7	100.3
	838.7	904.5 ± 67.1	7.4	107.8	907.0 ± 58.1	6.4	108.2
	1672.0	1729.3 ± 167.9	9.7	103.4	1717.9 ± 149.1	8.7	102.7
DCLZ	21.8	21.5 ± 2.4	11.3	98.5	21.6 ± 2.1	9.7	99.2
	47.4	48.5 ± 5.1	10.5	102.3	48.5 ± 5.0	10.4	102.3
	842.7	907.3 ± 54.0	5.9	107.7	913.3 ± 50.6	5.5	108.4
	1680.0	1719.8 ± 146	8.5	102.4	1733.3 ± 133.9	7.7	103.2
NTG	21.1	21.0 ± 1.6	7.7	99.5	21.2 ± 1.9	8.9	100.1
	45.6	49.1 ± 5.8	11.8	106.9	48.0 ± 5.7	11.9	104.4
	817.3	903.8 ± 59.0	6.5	110.6	908.1 ± 55.5	6.1	111.1
	1629.4	1724.6 ± 138.3	8.0	105.8	1715.5 ± 142.1	8.3	105.3

Table 4
Stability in rat plasma ($n = 6$)

Drug	Nominal concentration (ng/ml)	Sample condition							
		Bench top stability[a]		Autosampler stability[b]		Freeze–thaw stability[c]		30 days storage stability[d]	
		Nominal (%)	R.S.D.	Nominal (%)	R.S.D.	Nominal (%)	R.S.D.	Nominal (%)	R.S.D.
CLZ	47.2	103.1	6.2	107.8	8.9	102.4	7.1	97.5	8.6
	1672.0	99.3	2.0	103.5	6.1	98.7	1.7	98.0	2.2
DCLZ	47.4	102.0	5.9	98.9	11.1	100.6	5.9	97.1	8.8
	1680.0	99.0	2.0	108.0	6.4	99.1	1.8	97.2	1.9
NTG	45.6	98.9	7.6	103.7	11.6	97.1	6.6	95.6	8.0
	1629.4	99.3	1.5	106.3	6.0	99.8	0.9	98.7	1.4

[a] Exposed at ambient temperature (25 °C) for 6 h.
[b] Kept at autosampler temperature (10 °C) for 24 h.
[c] After three freeze–thaw cycles.
[d] Stored at −70 °C.

monitoring of anti-hyperglycemic agent, but involved gradient method. The aim of present investigation is to develop and validate a simple LC–MS/MS method using isocratic mode with sufficient accuracy and precision for simultaneous estimation of NTG and CLZ along with its active metabolite DCLZ and its subsequent use in pharmacokinetic studies in rats. The present method involves simple LLE procedure with good sensitivity and an isocratic reverse-phase HPLC analysis for all analytes of interest. This method is specific for NTG and CLZ and its major active metabolite DCLZ with no interference and with good linearity, accuracy and precision. This is also specific to repaglinide which was used as IS in this method. This method involves only 50 μl blood plasma and in our LLE extraction procedure we achieved a high level of extraction efficiency not only for CLZ, but also for its metabolite DCLZ as well as NTG too. This makes the assay highly reproducible and allows us to lower the limit of quantification. The LLOQ can be achieved to very low concentrations by increasing the plasma volume. Further more, this one-step LLE extraction procedure uses less single solvent in presence of 0.1N HCl, which decreases both the cost and duration of the assay. The chromatographic conditions of this method were optimized for a short 3.5 min run time on LC–MS/MS.

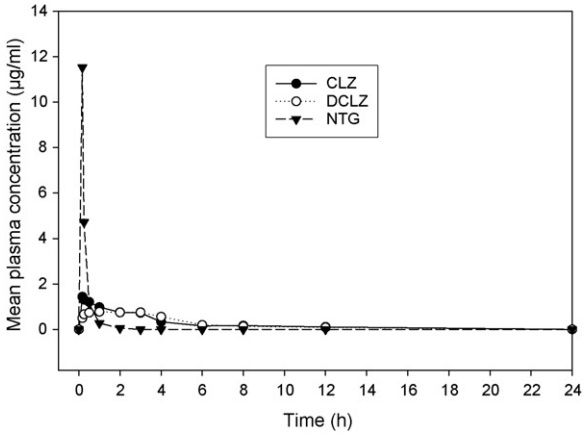

Fig. 3. Mean plasma concentration vs. time after single dose oral administration of NTG and CLZ in six Wistar rats.

Table 5
Pharmacokinetic parameters (mean ± S.D.) after single dose oral administration of NTG and CLZ simultaneously in Wistar rats

Parameters	Units	NTG	CLZ	DCLZ (metabolite)
C_{max}	μg/ml	11.53 ± 2.30	1.59 ± 0.39	0.93 ± 0.16
AUC_{0-24}	μg h/ml	2.85 ± 0.55	4.58 ± 0.69	4.31 ± 0.69
AUC_{0-inf}	μg h/ml	2.87 ± 0.54	5.12 ± 0.891	4.76 ± 0.72
T_{max}[a]	h	0.17 ± 0.00	0.25 ± 0.72	2.00 ± 1.02
$t_{1/2}$[b]	h	0.19 ± 0.11	3.26 ± 1.08	3.29 ± 1.66
K_{el}	h^{-1}	3.630 ± 1.502	0.213 ± 0.053	0.211 ± 0.076

[a] Median.
[b] Harmonic mean.

3.6. Application to pharmacokinetic study

The method described above was successfully applied to a PK drug–drug interaction study in which plasma concentration of pure markers was determined for up to 24 h after simultaneous oral administration at 10 mg/kg doses of NTG and CLZ in male Wistar rats. The plasma concentration time profiles of NTG, CLZ and its active metabolite DCLZ are shown in Fig. 3, and could be traceable up to 2, 12 and 12 h, respectively. Pharmacokinetic parameters were calculated from the plasma concentration time data by non-compartmental analysis using WinNonlin (Pharsight Inc., USA, version 5.1). The pharmacokinetic parameters of NTG, CLZ and DCLZ were presented in Table 5.

4. Conclusion

An LC–MS/MS bioanalytical method for simultaneous determination of three analytes, NTG, CLZ and DCLZ was developed and validated in rat plasma. The method was good enough to detect low concentration of 20 ng/ml for all these analytes in 50 μl rat plasma and further can be improved by increasing the plasma volume. Repaglinide, a meglitinide derivative with similar structural nucleus to NTG was used as IS in assay method to account the variations due to matrix effect, extraction variability and instrument performance. Analytes recovery from spiked

control samples were >90% except NTG where recovery was >75% using simple, convenient and fast LLE method. Intra- and inter-day accuracy and precision of the validated method were within the acceptable limits of <15% at low and <10% at other concentrations. The method was successfully applied to generate stability profile as well as PK evaluation of simultaneous administration of NTG, CLZ in rat following oral administration.

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