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Simultaneous determination of nitrendipine and hydrochlorothiazide in spontaneously hypertensive rat plasma using HPLC with on-line solid-phase extraction

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ABSTRACT

A HPLC method with on-line solid phase extraction (SPE) and DAD detection was developed for the simultaneous determination of nitrendipine and hydrochlorothiazide in spontaneously hypertensive rat (SHR) plasma. Plasma samples (100 μL) were injected directly onto a CAPCELL MF C_8 SPE column. High-abundance proteins and most matrixes in plasma were removed by on-line SPE technology, while nitrendipine and hydrochlorothiazide trapped on the SPE column were effectively separated on a C_{18} analytical column. The column temperature was maintained at 20 °C. The optimal detection wavelength was 237 nm for NTDP and 271 nm for HCTZ. The total analytical run time was 34 min. The proposed method was linear over the range 5–500 ng mL^{-1} for nitrendipine and 10–1000 ng mL^{-1} for hydrochlorothiazide. The lower limit of detection (LLOD) was 0.5 and 0.6 ng mL^{-1} for nitrendipine and hydrochlorothiazide, respectively. The sensitivity and precision of the method were within acceptable limits during validation period. The method was successfully used to investigate the pharmacokinetic characteristics of nitrendipine and hydrochlorothiazide in spontaneously hypertensive rats.

1. Introduction

Nitrendipine (NTDP, Fig. 1A), a dihydropyridine-type calcium antagonist, is widely used to reduce blood pressure in patients with mild or moderate hypertension, especially in elderly patients. The systemic bioavailability of nitrendipine is 14–20% due to first-pass metabolism, which may result in substantial inter-subject pharmacokinetic variability [1]. Despite the efficacy of NTDP, some patients with more resistant forms of hypertension may require an additional antihypertensive drug, such as hydrochlorothiazide (HCTZ, Fig. 1B), the most common diuretic in combination therapy for hypertension, to achieve adequate blood pressure control, especially for those with severe hypertension [2–4]. The combination of these two drugs is effective [5,6] in lowering blood pressure to desirable level with few side effects [7–9]. The combination of low-dose HCTZ and the calcium channel blocker NTDP is the first-choice therapy for community hypertensive patients in China [10].

HPLC methods with DAD [11,12], electrochemical [13–15] or LC–MS detection [16–18] have been developed for determination of either NTDP or HCTZ separately. Simultaneous determination of two compounds in the tablets by HPLC–DAD has been published [19], but the qualification limit of which was too high to meet the requirements of the biological fluids analysis. To date, no method for the simultaneous determination of NTDP and HCTZ in biological fluids has been reported. This may be because of the difficulties in extracting and detecting the two active ingredients simultaneously in plasma owing to their different polarity and solubility. Therefore, it is necessary to establish a simple analytical method for sample testing and quantitative research. In addition, an analytical method suitable for pharmacokinetic and drug–drug interaction studies of NTDP and HCTZ combinations is required.

Sample preparation is a crucial step in the analysis of biological samples. Solid-phase extraction (SPE) is one of the most practical techniques for sample enrichment as it overcomes many of the disadvantages in liquid–liquid extraction. Analysis of either NTDP or HCTZ in plasma using off-line SPE, which is widely applied to remove interfering biomatrix substances, has been reported [20,21]. However, the sample preparation is time-consuming

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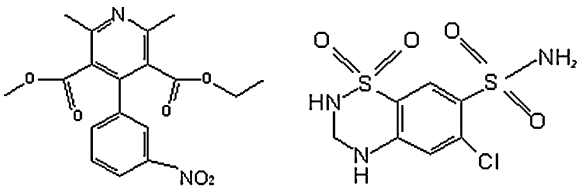

Fig. 1. Chemical structures of nitrendipine and hydrochlorothiazide.

and a new SPE cartridge must be used for each sample. Unlike liquid–liquid extraction, on-line SPE [22] has great advantages in permitting direct injection of plasma samples after short high-speed centrifugation or Millipore filtration. Furthermore, lower drug concentrations can be detected by increasing of the plasma injection volume. This technique delivers a simple, rapid and accurate means for determining analytes at low concentrations in real samples. This method has already been successfully used for analysis of similar compounds such as nifedipine [23]. LC–MS following on-line SPE could be the best choice in terms of sensitivity and specificity, but LC–MS is not available in many laboratories for economic reasons.

In the present study, we developed a simple and selective on-line SPE method for the simultaneous determination of NTDP and HCTZ in spontaneously hypertensive rat (SHR) plasma. An automated column exchange SPE system coupled to a HPLC system with DAD detection was optimized. The results show that this provides a sensitive, reliable and high-throughput method for the quantitative determination of NTDP and HCTZ in SHR plasma, and the method has great potential for preclinical, clinical, pharmacokinetic and drug–drug interaction studies for these two drugs.

2. Experimental

2.1. Chemicals and reagents

NTDP (98%) was purchased from Kehui Pharmaceutical Technology (Jinan, China). HCTZ (98%) was purchased from Yuancheng Technology Development (Wuhan, China). Methanol, acetonitrile, formic acid and other solvents were HPLC grade and were purchased from Fisher Scientific (USA). Pure water was obtained using a Nanopure water purification system (Thermo Fisher Scientific, USA). All other chemicals used in the study were analytical grade.

2.2. Instruments

The set-up comprised a Dionex (USA) Ultimate 3000 system equipped with DGP-3600A dual-gradient pumps (left pump and right pump, Fig. 2), an SRD-3600 solvent rack with integrated vacuum degasser, a WPS-3000TSL autosampler equipped with a 100-μL loop, a TCC-3200 thermostatted column compartment with a two-port, six-port (2P-6P) valve, a DAD detector and a Chromeleon chromatography data system. An ACCLAIM C_{18} (4.6 mm × 250 mm, 5 μm, 120 Å, Dionex, USA) column was used as the analytical column. CAPCELL MF C_8, CAPCELL PAK MF Ph-1 and CAPCELL PAK MF SCX columns (all 4.0 mm × 10 mm, 5 μm, Shiseido, Japan) were tested as the SPE column for analyte loading. The mobile phase A was pure acetonitrile and the mobile phase B was aqueous formic acid (4 mM), pH 3. The mobile phases A and B were for both left pump and right pump. The column temperature was maintained at 20 °C. The optimal detection wavelength was 237 nm for NTDP and 271 nm for HCTZ.

2.3. Stock solutions and calibration standards

Stock solutions of NTDP and HCTZ were prepared separately in acetonitrile at 100 μg mL^{-1}. These were further diluted with acetonitrile to concentrations of 50, 100, 200, 500, 1000, 2000 and 5000 ng mL^{-1} for NTDP and 100, 200, 500, 1000, 2000, 5000 and 10000 ng mL^{-1} for HCTZ. Plasma calibration standards containing 5, 10, 20, 50, 100, 200 and 500 ng mL^{-1} NTDP and 10, 20, 50, 100, 200 500 and 1000 ng mL^{-1} HCTZ were prepared by adding 20 μL NTDP and 20 μL HCTZ working stock solutions into 160 μL of blank SHR plasma. Quality control (QC) samples were prepared by spiking blank SHR plasma with independently prepared standard stock solutions to give final concentrations of 10, 50 and 200 ng mL^{-1} NTDP and 20, 100 and 500 ng mL^{-1} HCTZ. The calibration standards and QC samples were stored at −80 °C, thawed and filtered through a 0.22-μm membrane before injection on the on-line SPE–HPLC system.

2.4. Extraction procedure and chromatography for on-line analysis

A sample volume of 100 μL was directly injected for on-line SPE column switching and HPLC–DAD analysis. The on-line SPE–HPLC system is illustrated in Table 1 and Fig. 2.

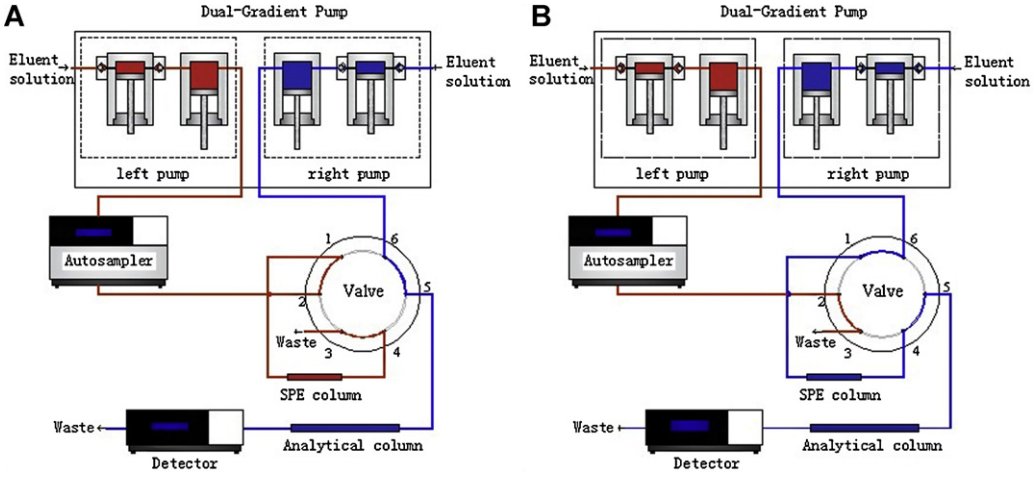

Fig. 2. Schematic of devices for the determination of nitrendipine and hydrochlorothiazide by on-line SPE–HPLC with DAD detection. (A) Sample was loaded onto the SPE column and (B) sample was desorbed from the SPE column and flowed onto the analytical column for quantification.

Table 1
Gradient program and valve switching.

Time (min)	Left pump (B, %)	Right pump (B, %)	Valve location
0	100	80	1_2
1.4	100	80	6_1
2.5	100	80	1_2
9	100	80	1_2
10	100	40	1_2
10.5	100	40	6_1
15	100	40	1_2
24	100	40	1_2
25	100	80	1_2
34	100	80	1_2

- Step 1 (Fig. 2A). With the six-port injector valve in the 1_2 position, 100 μL of plasma sample was directly injected into the system. The mobile phase B at a flow rate of 1.0 mL min^{-1} for ~1.4 min was used to wash out high-abundance proteins, while NTDP and HCTZ were trapped on the SPE column.
- Step 2 (Fig. 2B). The six-port valve was switched to the 6_1 position to connect the SPE column and the analytical column in series. HCTZ was eluted from the SPE column onto the analytical C_{18} column with the mobile phase A/the mobile phase B (20:80, v/v) at an optimized flow rate of 1 mL min^{-1} for 1.1 min. At the same time, NTDP was still trapped on the SPE column.
- Step 3 (Fig. 2A). The six-port valve was switched back to the 1_2 position and residual trace matrix material on the SPE column was flushed to waste using the same eluent as above for 8 min.
- Step 4 (Fig. 2B). The six-port valve was switched to the 6_1 position to connect the SPE column and the analytical column in series again. NTDP was eluted from the SPE column onto the analytical column using the mobile phase A/the mobile phase B (60:40, v/v) at a flow rate of 1 mL min^{-1} for 4.5 min.
- Step 5 (Fig. 2A). The six-port valve was switched back to the 1_2 position and the SPE column was prepared for the next sample while the analytical column continued to separate the analytes.

2.5. Method validation

2.5.1. Selectivity and specificity

Interference by endogenous compounds in plasma was assessed by comparing chromatograms of blank SHR plasma, plasma spiked with NTDP and HCTZ, and plasma samples obtained from pharmacokinetic studies.

2.5.2. Linearity

Calibration curves were constructed from the working standard solutions for the range 5–500 ng mL^{-1} for NTDP and 10–1000 ng mL^{-1} for HCTZ. The peak areas for NTDP and HCTZ were plotted against the spiked NTDP and HCTZ theoretical concentrations in blank plasma. Least-squares linear regression was used for curve fitting with $1/C$ as the weighting factor.

2.5.3. Sensitivity

The lower limit of detection (LLOD) is the level at which the analyte amount provides a signal of three times the background noise. Similarly, the lower limit of quantification (LLOQ) corresponds to a signal/noise ratio of ten.

2.5.4. Precision and accuracy

Precision was evaluated by measuring intra- and inter-day relative standard deviations (R.S.D.). The intra-day precision was performed by assaying five spiked samples on a single day at three different concentrations (QCs) for NTDP and HCTZ. The inter-day precision was performed by analyzing spiked samples at three different concentrations for NTDP and HCTZ on five consecutive days. The precision criterion for the acceptability of data was that the variation for each concentration level should not be greater than ±15% deviation from the nominal values except for LLOQ, for which it should not exceed ±20%. Similarly, for accuracy, the mean value should not deviate by ±15% from the nominal concentration except for LLOQ, for which the limit was ±20%.

2.5.5. Recovery

The extraction recovery for the analytes for three QC samples was assessed by comparing the peak areas for extracted spiked plasma samples with the peak areas for pure compounds of the same concentrations in solvent.

2.5.6. Stability

Short-term, long-term and freeze–thaw cycle stability was assessed by analyzing the three QC samples for five replicates. The QC samples were analyzed after storage at 4 °C for 12 h and at −80 °C for 1 month. Freeze–thaw stability was evaluated after three cycles (−20 °C/room temperature). Deterioration for each analyte was defined as a greater than 15% difference in the test sample versus the control at the nominal sample concentration.

2.5.7. Pharmacokinetic study

SHR animals (250 ± 50 g) were obtained from Vital River Laboratories (Beijing, China). Animal pharmacokinetic study had been approved by the Ethic Committee of Animal Experiments of Peking University. Suspensions of NTDP and HCTZ were prepared in 0.8% sodium carboxymethylcellulose by ultrasonic shaking until a uniform solution was obtained. For pharmacokinetic studies, the prepared suspension was orally administered to six rats after an overnight fast at a dose of 10 mg kg^{-1} for NTDP and 20 mg kg^{-1} for HCTZ. The dose was determined by reference to previous studies on these two drugs [24]. Plasma was separated from blood by centrifugation at 10,000 rpm for 10 min and was stored in a freezer (−80 °C) until analysis.

3. Results and discussion

In the present study, on-line SPE with HPLC and DAD detection was selected owing to its sensitivity, selectivity and convenience.

3.1. SPE optimization

High-throughput analysis using on-line SPE requires a high solution loading speed to purify and concentrate target compounds. It was found that different types of SPE column had no influence on the NTDP retention behavior and peak shape. However, HCTZ was hardly retained on some of the SPE columns owing to its hydrophilicity. Thus, the retention behaviors of NTDP and HCTZ during on-line SPE were investigated using CAPCELL PAK MF Ph-1, CAPCELL PAK MF SCX and CAPCELL PAK MF C_8 SPE columns. The retention specificity of HCTZ on the CAPCELL PAK MF C_8 column was better than on the other two columns (Fig. 3), so this was chosen for sample loading.

3.2. Optimization of analyte transfer time

The analyte transfer time from the SPE column to the analytical column via valve switching is crucial. If the time is too short, analyte recovery can be poor. If the time is too long, endogenous impurities might be transferred to the analytical column, which could affect the long-term performance of the assay. During the 34-min cycle, a transfer time of 1.4–2.5 min for HCTZ and 10.5–15.0 min for NTDP was found to be suitable for the assay. The gradient program and valve switching were controlled by a computer, as shown in Table 1.

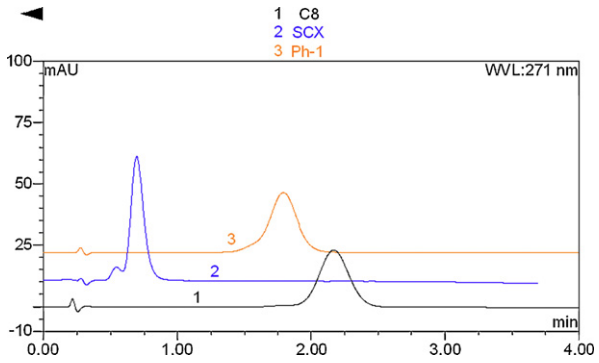

Fig. 3. The retention behaviors for hydrochlorothiazide in different SPE columns. (A) CAPCELL PAK MF C8 SPE column, (B) CAPCELL PAK MF SCX SPE column and (C) CAPCELL PAK MF Ph-1 SPE column.

The total analytical run time was 34 min. The SPE column can be used repeatedly for more than 100 samples.

3.3. Method validation

3.3.1. Selectivity and specificity

Blank plasma was collected from intact SHRs, then extracted and analyzed as stated above. Representative chromatograms of blank SHR plasma, blank SHR plasma spiked with NTDP (500 ng mL^{-1}) and HCTZ (500 ng mL^{-1}), and SHR plasma collected at 0.5 h after oral administration of NTDP (10 mg kg^{-1}) and HCTZ (20 mg kg^{-1}) are shown in Fig. 4. The identity of nitrendipine and hydrochlorothiazide in the endogenous samples was demonstrated by using mass-spectrometric methods (figure not shown). No significant interferences by endogenous substances were observed at the retention times for NTDP and HCTZ.

3.3.2. Linearity

Calibration curves for the method covered the range 5–500 ng mL^{-1} for NTDP and 10–1000 ng mL^{-1} for HCTZ. Using weighted least-squares (WLS) regression with a statistical weight of $1/C$, calibration equations $A = 0.0064 \times C - 0.0088$ and $A = 0.0050 \times C + 0.0004$ were obtained for NTDP and HCTZ, respectively. The goodness of fit for both NTDP and HCTZ was consistently greater than 0.99 during validation.

3.3.3. LLOD and LLOQ

In this study, the LLOD was 0.5 ng mL^{-1} for NTDP and 0.6 ng mL^{-1} for HCTZ and the LLOQ was 5 ng mL^{-1} for NTDP and 10 ng mL^{-1} for HCTZ in 100 μL of plasma. The theoretical LLOQ is approximately 2 ng mL^{-1}, which is similar to that of other HPLC–UV methods [20,21,23]. Only LC–MS–MS analysis has greater sensitivity, but the equipment is very expensive and is not available in all clinical laboratories. Samples with concentrations below the LLOQ, such as elimination phase samples, could be determined by increasing the injection volume. This is a great advantage of on-line SPE methods; lower concentrations can be detected by increasing the injection volume without increasing impurities. Therefore, this method is suitable for analysis of NTDP and HCTZ in pharmacokinetic studies.

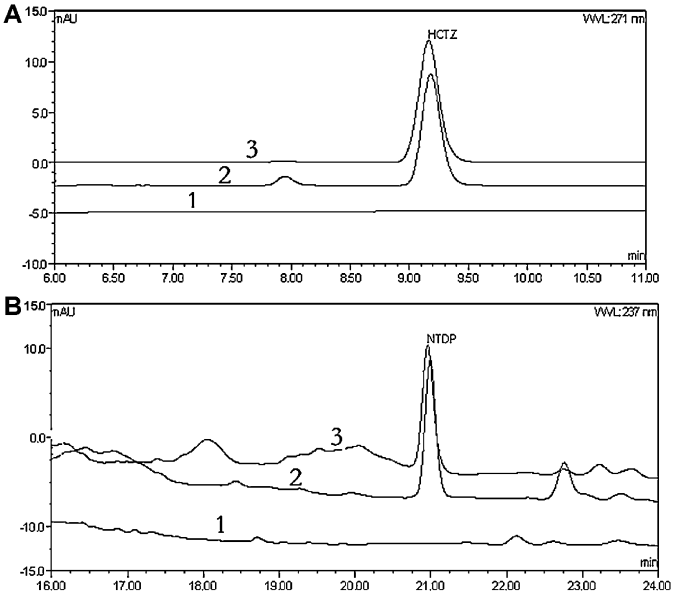

Fig. 4. The HPLC chromatograms for hydrochlorothiazide (A) and nitrendipine (B) in SHR plasma: blank SHR plasma (A-1 and B-1), blank SHR plasma spiked with hydrochlorothiazide (500.0 ng mL^{-1}, A-2) and nitrendipine (500.0 ng mL^{-1}, B-2), and hydrochlorothiazide (A-3) and nitrendipine (B-3) in a plasma sample from a SHR at 0.5 h after an oral administration.

3.3.4. Precision and accuracy

Table 2 shows a summary of the intra- and inter-day precision and accuracy. The intra- and inter-day accuracy was within ±15% (85–115%) for all QC samples for both analytes. The intra- and inter-day assay precision (R.S.D.) was also within the acceptable range of 15% for all samples. The QC data illustrate the stability and reliability of this method for determination of HCTZ and NTDP in SHR plasma.

3.3.5. Recovery

The proposed method simplified the sample preparation step, effectively enriched the analytes and cleaned up endogenous compounds. The mean recovery from SHR plasma was 94.97%, 90.09% and 85.76% for HCTZ and 91.69%, 103.06% and 95.13% for NTDP for low, medium and high QC samples, respectively.

3.3.6. Stability

Stability results for HCTZ and NTDP under various conditions are summarized in Table 3. The accuracy for HCTZ and NTDP ranged from 92.02% to 103.9%. Thus, the method is suitable for

Table 2
Intra- and inter-day accuracy and precisions of the hydrochlorothiazide and nitrendipine in SHR plasma ($n = 5$).

Analytes	Nominal concentration (ng mL^{-1})	Intra-day			Inter-day		
		Mean ± S.D.	Accuracy (%)	R.S.D. (%)	Mean ± S.D.	Accuracy (%)	R.S.D. (%)
HCTZ	20	21.04 ± 0.21	105.20	1.00	20.99 ± 0.27	104.95	1.29
	100	96.20 ± 1.62	96.20	1.68	97.26 ± 2.72	97.26	2.80
	500	472.04 ± 17.97	94.41	3.81	461.80 ± 19.95	92.36	4.32
NTDP	10	9.20 ± 0.18	92.00	1.96	9.33 ± 0.32	93.30	3.43
	50	52.83 ± 1.48	105.66	2.80	53.57 ± 3.51	107.14	6.55
	200	200.52 ± 5.01	100.26	2.50	201.40 ± 5.82	100.70	2.89

Table 3
Stability results of the analytes in spiked plasma samples ($n = 5$).

Analytes	Nominal concentration ($ng\,mL^{-1}$)	Short-term stability (12 h at 4 °C)			Freeze–thaw stability (three cycles)			Long-term stability (1 month at −80 °C)		
		Mean ± S.D.	R.S.D. (%)	Accuracy (%)	Mean ± S.D.	R.S.D. (%)	Accuracy (%)	Mean ± S.D.	R.S.D. (%)	Accuracy (%)
HCTZ	20	20.52 ± 0.96	4.68	102.60	21.84 ± 1.27	5.82	109.2	20.78 ± 1.21	5.82	103.90
	500	465.89 ± 24.75	5.31	93.18	469.79 ± 21.10	4.49	93.96	460.10 ± 18.65	4.05	92.02
NTDP	10	9.33 ± 0.32	3.43	93.30	9.47 ± 0.54	5.70	94.70	9.23 ± 0.72	7.80	92.3
	200	199.74 ± 9.78	4.90	99.87	200.02 ± 10.64	5.32	100.01	197.32 ± 14.76	7.48	98.66

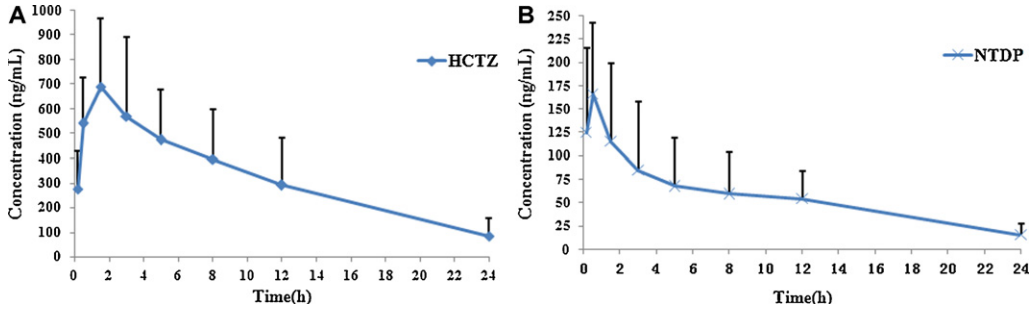

Fig. 5. Plot of mean concentrations versus time in SHRs by oral administration of 10 mg kg^{-1} nitrendipine (A) and 20 mg kg^{-1} hydrochlorothiazide (B) ($n = 8$; Mean ± S.D).

pharmacokinetic studies of HCTZ and NTDP in SHR plasma. No significant change in NTDP or HCTZ concentrations in SHR plasma was observed during the 12-h assay period. Freezing and thawing did not have any detrimental effect on absolute concentrations of analytes spiked in SHR plasma. No obvious trend for concentration changes in NTDP or HCTZ was observed during storage at −80 °C.

Most of dihydropyridine-type calcium antagonists were photodegradative, so plasma sample handling should not be carried out with exposure to daylight for a long time. The concentration profiles of NTDP is fitted to a zero-order kinetic and the concentration of NTDP will decrease about 5% in 12 h UV lighting (254 nm) [25]. In this study, plasma samples were directly injected into the on-line SPE column, which minimized the influence of light on the stability of NTDP.

3.4. Application to pharmacokinetic study

The proposed method was used to analyze real plasma samples obtained from SHRs after simultaneous oral administration of NTDP (10 mg kg^{-1}) and HCTZ (20 mg kg^{-1}). By interpolating peak areas on the calibration curves, mean plasma concentration–time curves were constructed for the two analytes, as shown in Fig. 5. After oral dosing, NTDP showed rapid oral absorption in SHRs, with a short T_{max} of 30 ± 6 min. C_{max} and AUC_{0-t} for NTDP were approximately 146.9 ± 48.2 ng mL^{-1} and 976 ± 612 ng mL^{-1} h kg^{-1}, respectively. The T_{max}, C_{max} and AUC_{0-t} for HCTZ were 2.8 ± 1.7 h, 775.9 ± 253.9 ng mL^{-1} and 7703 ± 2343 ng mL^{-1} h kg^{-1}, respectively. The pharmacokinetic parameters of HCTZ determined in this study were similar to the published values in the rats administered alone [26]. But AUC of NTDP was different from the published values because of various administration routes and dosage forms [27].

4. Conclusion

In this study, a selective and sensitive procedure involving on-line SPE with HPLC was developed for determination of HCTZ and NTDP in SHR plasma.

The detection limit means that this method is suitable for determining terminal phase concentrations of NTDP and HCTZ after oral administration of lower doses. With a much larger volume of human plasma, lower concentrations could be determined using the system. This would widen the scope of the application of the on-line SPE HPLC–DAD technique. Furthermore, the method is suitable for preclinical and clinical research.

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