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Original Article

THE MEASUREMENT OF CEFOTAXIME SODIUM IN RAT PLASMA AFTER ORAL ADMINISTRATION: A SENSITIVE HPLC-UV METHOD

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ABSTACT

Objective: To develop and validate a high-performance liquid chromatographic method for the determination of cefotaxime sodium (NaCTX) concentration in rat plasma.

Methods: The method used direct injection of the plasma supernatant after deproteinization with perchloric acid. Degradation of NaCTX in acidic medium during sample treatment was retarded by reducing the strength of perchloric acid to 30%, followed by adding 0.55M of aqueous disodium hydrogen orthophosphate buffer before centrifuging the sample. The mobile phase used was consisted of 0.04M aqueous ammonium acetate: acetonitrile: tetrahydrofurane (90:7:3, v/v) with pH adjusted to 5.7 using glacial acetic acid. The flow rate was 0.9 ml/min, UV detector set at 254 nm and samples were quantified using peak area

Results: A well-resolved NaCTX peak and free of interference from endogenous compounds in rat plasma were achieved. Recovery of NaCTX was satisfactory over the concentration range tested 0.125-10 µg/ml. limit of quantification (LOQ) of this assay was 0.125 µg/ml and, at this concentration, intra-and inter-day CV were 5.33 and 6.13 %, respectively. NaCTX was found to be stable in rat plasma after storage at-80 °C, over 90 days. The plasma concentration-time profile in rats for NaCTX after oral administration of NaCTX solution was achieved using the present method.

Conclusion: The stability, sensitivity, specificity and reproducibility of this method make it suitable for the determination of NaCTX plasma concentration in pharmacokinetics and bioavailability studies.

Keywords: Cefotaxime sodium, HPLC-UV assay, Validation, Stability, Plasma concentration.

INTRODUCTION

Cefotaxime sodium (NaCTX) is a third-generation cephalosporin, which exhibits potent activity against many gram-negative and gram-positive organisms [1]. It contains an amino thiazolyl side chain, with an alpha-methoxyimino group at position 7 of the cephalosporin nucleus (fig. 1).

According to British Pharmacopeia (2005), the empirical formula for NaCTX is $C_{16}H_{17}N_5NaO_7S_2$ and the molecular weight is 477.468 g/mol. NaCTX contains approximately 50.5 mg (2.2 mEq) of Na per g of CTX.

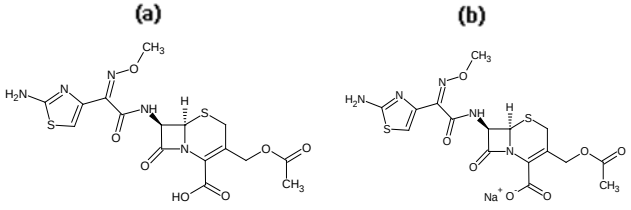

Fig. 1: Chemical structures of (a) cefotaxime (CTX) and (b) cefotaxime sodium (NaCTX)

Drug content, concentration, loading and kinetic release in relation to NaCTX can be addressed using *in vitro* and *in vivo* studies. Assays based on reversed-phase high performance liquid chromatography (HPLC) with detection by ultraviolet (UV) or mass spectrometry (MS) are commonly applied. Validation of the analytical methods for NaCTX must be conducted to determine a number of parameters, such as specificity, sensitivity, linearity, limit of quantification (LOQ), limit of detection (LOD), accuracy, precision, recovery and drug stability in the sample.

A number of HPLC analytical methods have been developed for the determination of CTX in biological samples [2-9]. They generally were used a reversed-phase systems with UV detection but a HPLC run time for drug analysis was 20-40 minutes [2-7]. Moreover, the sensitivity of most reported methods is rather poor [3, 6-8, 10-14], using peak height quantification rather than peak area and various organic solvents or strong acid were used, such as 70% perchloric acid for deproteinizing the CTX in plasma samples. However, no information regarding long term stability is available in many studies. Most of the reported methods though were not sufficiently validated. Thus, in this paper a sensitive, selective, reproducible and stability indicating HPLC-UV assay of NaCTX concentration in rat plasma was developed and validated for accuracy, precision, recovery, linearity and long-term stability. This developed method was verified by analyzing plasma samples from rats after an oral administration of NaCTX solution.

MATERIALS AND METHODS

Materials

Chemicals (suppliers) were as follows: NaCTX (Sigma Aldrich Co, St Louis, USA); HPLC grade perchloric acid; ammonium acetate and sodium dihydrogen orthophosphate (Ajax Chemicals, Mulgrave, Australia); disodium hydrogen orthophosphate (Ajax Finechem, Auckland, New Zealand); HPLC grade acetonitrile, chloroform, tetrahydrofuran (THF) and methanol (Merck, Darmstadt, Germany); Heparin sodium (CP Pharmaceutical Ltd, Wrexham, UK); Normal Saline Solution (Pfizer, NY, USA).

Methods

HPLC system for plasma samples

A complete Shimadzu HPLC system (Shimadzu Corporation, Japan) was used consisting of a LC-10A vp Intelligent HPLC pump and a SPD-10A vp detector set at an operation wavelength of 254 nm and a

Rheodyne 7725i injector fitted with a 50 µl sample loop (Rheodyne, USA). This HPLC system was typically used to analyze all the NaCTX plasma samples and all obtained data were collected and processed by Shimadzu Class VP version 7.4 software. A Phenomenex SB-C18 reversed phase analytical column (150 x 4.6 mm, 5 µm) (Torrance, CA, USA) fitted with Refillable Phenomenex guard column (10 x 2 mm, 5 µm) packed with Perisorb RP-18 (Upchurch Scientific, Oak Harbour, WA, USA) was used with mobile phase of 0.04M aqueous ammonium acetate: acetonitrile: THF (90:7:3, v/v), adjusted to pH 5.7 using glacial acetic acid. Data manipulation and processing was carried out using EZC from Elite software. The flow rate was 0.9 ml/min and the samples were quantified using peak area.

Rat treatment

NaCTX solution (50 mg/ml) for oral administration was prepared by adding 250 mg drug powder to 5 ml normal saline solution and stirring thoroughly at room temperature for 1 h. It was stored at +4 °C to+8 °C in the fridge and used within 12 h of preparation. NaCTX was administered to the rats orally by gavage in the dose of 50 mg/kg. Five male Wister rats (age 2-3 mo, weight 300 g) were used in the experiment. They were kept in the experimental animal facility and given the standard diet and water *ad libitum*. Temperature and light were controlled mimicking the natural habitat. The study was approved by Medical Faculty Novi Sad Animal Ethics Committee.

Blood samples were collected into heparinized tubes from the tip of the tail pre-dose and at 15, 30, 60, 90, 120, 150, 180 and 240 min after the dose.

Blood samples were centrifuged at 3,000 g for 15 min at+4 °C to collect plasma which was stored at -80 °C pending HPLC analysis. Immediately after the last blood sample, rats were sacrificed by carbon dioxide asphyxiation. Samples were analyzed within 6 days of collection.

Sample preparation

Prior to analysis, NaCTX was extracted from plasma samples as follows: aliquot samples of plasma (100 µl) were transferred into Eppendorf micro-centrifuge tubes and deproteinized by adding 15 µl aliquots of 30% w/w perchloric acid. Samples were vortex-mixed for 30 s (Stuart Scientific, UK) after which,70 µl of 0.55M aqueous disodium hydrogen orthophosphate buffer was added immediately and the mixture vortexed for a further 15 s. Samples were then centrifuged (Eppendorf, Hamburg, Germany) at 15,000 g for 10 min and 60 µl of the supernatants injected into the HPLC system.

Assay validation

A stock solution of NaCTX (1 mg/ml) was prepared in water and stored at-80ºC until required. Three different batches of six standard curves were prepared by spiking rat plasma with NaCTX solution, spiking Mobile phase (MP) with NaCTX solution, spiking water with NaCTX solution to give concentrations in the range 0.125-10µg/ml. QC samples (0.125, 1, 3 and 6 µg/ml NaCTX) were prepared by spiking rat blank plasma with NaCTX solution standards in exactly the same way. The calibration standards were stored at-80ºC until used for assay validation and sample analysis. Linearity was assessed by least–square regression and lack-of-fit analysis (Minitab 15. Accuracy was expressed as the percentage of the spiked concentration and precision as the coefficient of variation (CV). LOQ is defined as the concentration with signal-to-noise ratios of 10:1. For inter-day, accuracy and precision determinations, analyses of each concentration were carried out daily for 6 consecutive days, whereas for intra-day accuracy and precision evaluations, analyses were carried out 6 times on the same day. Recovery was estimated by comparing peak areas in QC samples (n=6) with drug solutions at corresponding concentrations. The stability of NaCTX was tested in plasma at 4 different concentrations (0.25, 2, 4 and 8 µg/ml NaCTX); Short term stability (STS) at-80 °C was tested over 2 days while long term stability (LTS) was tested at -80 °C over 30 and 90 days.

Statistical analysis

Analysis of variance (one-way ANOVA) was used to compare mean values of variables determined for different standards curves spiked with different Martials. Differences were considered statistically significant when *$p<0.05$. Analysis was performed using the Minitab program (Version 15; Minitab Inc, USA).

RESULTS

Chromatograms of water spiked with 5µg/ml NaCTX, plasma spiked with 5µg/ml NaCTX and plasma free of NaCTX are shown in fig. 2. The NaCTX peak with a retention time of approximately 8.8 min was well-resolved and free from interference from endogenous compounds in rat plasma.

The total run time for each sample was 10 min. At least six batches of rat plasma were tested. Thus, blank plasma was used in the preparation of the calibration curves. NaCTX was found to be stable in plasma for at least 18 h at room temperature with no noticeable changes in NaCTX-plasma concentrations.

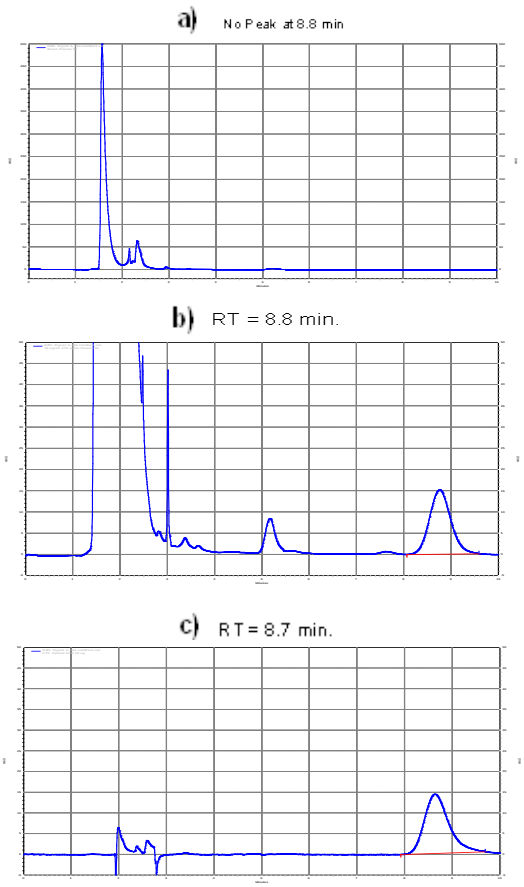

Fig. 2: Chromatograms of (a) blank rat plasma, (b) blank rat plasma spiked with 5 µg/ml NaCTX and (c) blank water spiked with 5 µg/ml NaCTX

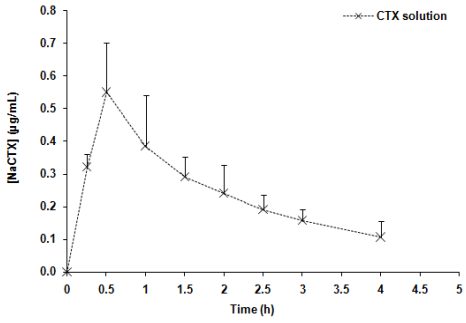

Fig. 3: Plasma concentration-time profiles of NaCTX after oral administration (50 mg/kg) of NaCTX solution to male Wister rats (data are means±S. D., n = 5).

Table 1: Comparison between r^2, slopes and intercepts of different standard curves (n=6) (CTX-HPLC assay)

St Curves	Regression r^2	Slope (mean±SE)	Intercept (mean±SE)
Plasma spiked with NaCTX	0.9996±0.0006	1.0029±0.24	+0.0316±0.0298
Water spiked with NaCTX	0.9999±0.0011	1.0489±0.36	-0.1104±0.0834
MP spiked with NaCTX	0.9998±0.0058	1.6874±0.41	-0.0135±0.0312

The calibration curve of NaCTX spiked with rat plasma, water and MP (n=6) as values reported in table 1, were linear over the concentration range 0.125-10 µg/ml. (r^2 0.9996±0.0006) with a mean intercept of 0.0316±0.0298. Similarly, the calibration curve of NaCTX spiked with water and MP (table 1) was linear over the same concentration range (r^2 0.9999±0.0011 and 0.9998±0.0058, respectively) with a mean intercept of-0.1104±0.0834 and-0.0135±0.0312, respectively. It is evident that a linear correlation is existed between the peak area and the concentrations of NaCTX spiked with plasma and with water/MP. This result also demonstrates that no considerable interference is existed as no significant deference was found (*p>0.05*).

Table 2: Measured NaCTX, Intra-day accuracy and precision of the HPLC method for the analysis of NaCTX in rat plasma samples (data are means±SD, n=6)

Nominal [NaCTX] (µg/ml) Intra-day	Measured [NaCTX] (µg/ml)	Accuracy (%)	Precision (CV) (%)
0.125	0.121±0.31	95.21	5.33
1.00	0.98±0.40	101.40	3.15
3.00	2.95±1.10	102.18	5.52
6.00	5.97±1.35	96.77	2.59

Table 3: Measured NaCTX, Inter-day accuracy and precision of the HPLC method for the analysis of NaCTX in rat plasma samples (data are means±SD, n=6)

Nominal [NaCTX] (µg/ml) Inter-day	Measured [NaCTX] (µg/ml)	Accuracy (%)	Precision (CV) (%)
0.125	0.120±0.20	93.50	6.13
1.00	0.98±0.75	95.84	6.20
3.00	2.96±0.10	104.2	5.89
6.00	5.97±0.40	98.16	4.09

Measured NaCTX, Inter-and intra-day accuracy and precision values for different NaCTX concentrations are presented in table 2 and 3, respectively. LOQ as the lowest concentration used in the construction of the calibration curves was 0.125µg/ml and at this concentration, intra-and inter-day CV were 5.33% and 6.13%, respectively, whereas the recovery was>95%. This demonstrates that the present method was found to be more sensitive than previously reported methods [3, 6-8, 10-13].

Table 4: NaCTX stability in rat plasma after 2, 30 and 90 days at-80ºC (data are means±S. D., n=6)

Nominal [NaCTX] (µg/ml)	[NaCTX] at 2 days (µg/ml)	[NaCTX] at 30 days (µg/ml)	[NaCTX] at 90 days (µg/ml)
0.25	0.24±0.45	0.24±0.21	0.23±0.64
2.00	1.98±0.11	1.96±0.13	1.95±1.16
4.00	3.96±1.52	3.95±1.15	3.94±1.38
8.00	7.98±0.21	7.95±1.38	7.93±1.15

Furthermore, the present method was testing the NaCTX stability in plasma before extraction. For stability indicating method, NaCTX was found to be stable in rat plasma after storage at-80ºC for 2, 30 and 90 days (table 4). The result demonstrates that the assay is stable, selective and sufficiently sensitive for pharmacokinetic studies in rat.

For verification, the present developed method was used to analyze plasma samples from five rats after an oral administration of 50 mg/kg NaCTX solution. Fig. 3 shows the plasma concentration-time profiles for CTX (as NaCTX) over 4 h after the oral dose (50 mg/kg) of NaCTX solution. It is apparent that the present HPLC developed method was sensitive enough to detect the plasma concentration-time profiles for a drug with a well-known low oral bioavailability.

DISCUSSION

A number of analytical methods have been reported for determination of NaCTX in plasma [2, 3, 5-8, 13, 15, 16]. The HPLC method developed here was based on the method reported by Jehl [2], but with some modifications to the mobile phase ratios, concentration, flow rate of MP, pH, strength and volume of the deproteinizing agent. The concentration of the aqueous ammonium acetate in the buffer was increased from 0.02M to 0.04M and the pH was adjusted to 5.7 instead of 5.0. The addition of 3% THF was found to be necessary to produce sharp and well-resolved peaks free of interference from endogenous compounds.

NaCTX is susceptible to degradation in acidic pH [17]. Studies have shown that CTX exhibits maximum stability in solutions with pH in the range 4.3-6.5 [18, 19]. Degradation appears to be rapid at pH 1.5. It was reported that the β-lactam moiety undergoes hydrolysis at lower pH while at higher pH (>8), the side chain undergoes hydrolysis. Thus, for plasma sample preparation, the use of 70% perchloric acid as a deproteinizing agent was found to be critical and essential to produce a clear sample. But, using perchloric acid can lead to hydrolysis of NaCTX.

Using other organic solvents such as acetonitrile, methanol, THF and phosphoric acid were tried but they did not produce clear samples. They can induce turbidity after sample treatment. Therefore, 70% perchloric acid was used after the strength and the volume of the acid was reduced to 15µl of 30 %, to retard NaCTX degradation. 70 µl of 0.55 M aqueous disodium hydrogen orthophosphate was added immediately to the sample to increase the pH of the samples and to avoid further hydrolysis of NaCTX over sample preparations. It is evident that a linear correlation is existed between the peak area ratio of plasma spiked NaCTX and water/MP spiked NaCTX. This result also demonstrates that no considerable interference is existed. The extraction recovery of NaCTX was determined by comparing the peak area obtained by direct injection of standard aqueous solutions to those obtained after the plasma extraction procedure and it has been reported as a measured drug value. The used deproteinizing agent was found essential to give satisfactory recoveries for NaCTX and provide clearer chromatograms as compared to using other deproteinizing agents. LOQ of this assay was 0.125µg/ml. This demonstrates that present method was found to be more sensitive than previously reported methods [2-9]. For stability indicating, NaCTX was found to be stable in plasma after storage at-80 °C for 2, 30 and 90 days. No considerable differences were observed.

It is apparent that the plasma concentration of NaCTX after the oral administered dose was low, indicating very low oral bioavailability of NaCTX when administered as an aqueous solution of NaCTX. However, the present developed method was sensitive enough to detect the plasma concentration-time profiles for a drug with well-known low oral bioavailability (0.53-1.53 %).

CONCLUSION

In conclusion, the HPLC method described here is a simple, sensitive, selective, reproducible, linear, precise, accurate, stability indicating and requires only a small volume for the determination of NaCTX in rat plasma. The method is applicable for the determination of CTX *in vivo* and for the assessment of pharmacokinetic and oral bioavailability studies of NaCTX.

CONFLICT OF INTERESTS

Declared None

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