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Comparative Determination of Cefotaxime and Desacetyl Cefotaxime in Serum and Bile by Bioassay and High-Performance Liquid Chromatography

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Dedicated to Professor Dr. H. Oelschläger on the occasion of his 60th birthday

Summary: *In rat serum as well as in human serum and bile after injection of cefotaxime (CTX), the parent compound and the active metabolite desacetyl cefotaxime (dCTX) have been demonstrated by quantitative analysis with high-performance liquid chromatography (HPLC). Simultaneous determination of CTX by bioassay using test organisms sensitive to both CTX and dCTX resulted in spuriously high concentration readings of CTX. Using dCTX insensitive test organisms concentrations of cefotaxime obtained by agar diffusion test and by assay with HPLC were highly correlated. In human serum and bile after i.v. injection of 2 g CTX, as may be administered therapeutically, high concentrations of dCTX were observed. dCTX has a longer elimination half-life than the parent molecule. It is concluded that the measurement of CTX in biological fluids should be performed by HPLC or by bioassay with a selective test organism in order to obtain correct pharmacokinetic parameters.*

Zusammenfassung: *Vergleichende Bestimmung von Cefotaxim und Desacetylcefotaxim in Serum und Galle mit*

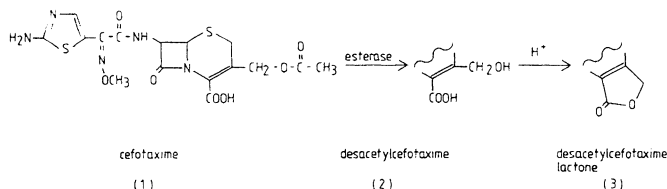
Agardiffusionstest und Hochdruckflüssigkeitschromatographie

In Serum von Ratte und Mensch sowie in menschlicher Galle wurde nach Injektion des Antibiotikums Cefotaxim (CTX) mit Hilfe der Hochdruckflüssigkeitschromatographie (HPLC) Cefotaxim und der mikrobiologisch aktive Metabolit Desacetylcefotaxim (dCTX) nachgewiesen. Mikrobiologische Bestimmungen von Cefotaxim mit sowohl gegen CTX als auch dCTX empfindlichen Testkeimen im Agardiffusions-Test (ADT) führten für Cefotaxim zu irrtümlich hohen Konzentrationen. Verwendung von gegen dCTX unempfindlichen Keimen im ADT ergab Konzentrationen, die mit den Werten der HPLC hoch korrelierten. In Serum und Galle des Menschen fanden sich nach Injektion einer therapeutischen Dosis von 2 g CTX hohe Konzentrationen sowohl von CTX als auch von dCTX. Die Halbwertszeit von dCTX ist länger als die von CTX. CTX sollte deshalb in biologischen Flüssigkeiten mit Hilfe der HPLC oder unter Verwendung von dCTX-unempfindlichen Testkeimen bestimmt werden, um richtige pharmakokinetische Daten zu erhalten.

Key words: *Antibiotics · Cefotaxime, bile concentrations, serum levels · Desacetyl cefotaxime*

1. Introduction

Cefotaxime (CTX) [1] is chemically characterized by a 2-amino-4-thiazolyl ring, which in comparison to cefuroxime increases antibacterial activity against gram-negative strains, and by an α -methoximino group like cefuroxime, which enhances stability to β -lactamases [3, 6, 7].



Furthermore, CTX has a 3-acetoxy-methyl substituent. It has been shown earlier that other cephalosporins with this substituent, e.g., cephalothin, are disintegrated to the respective desacetyl compounds [1].

Investigating serum concentrations of CTX in rats by a usual microbiological assay we obtained very high levels as calculated from the antibacterial activity observed.

Analysis of the serum concentration in the same samples by high-performance liquid chromatography (HPLC), however, resulted in significantly lower concentrations of CTX. This prompted us to search for an active metabolite of cefotaxime after parenteral application of the cephalosporin in man and rats.

2. Materials and methods

2.1. Chemicals

Cefotaxime (CTX), desacetyl cefotaxime (dCTX) and desacetyl cefotaxime lactone (dCTXL) were gifts from Hoechst Aktiengesellschaft, Frankfurt (M). Fresh stock solutions were prepared daily and serial dilutions made either with serum or bile.

2.2. Investigations in rats

Male Sprague-Dawley SPF rats (S. Ivanovas, D-7967 Kißlegg/Allgäu) weighing 100 ± 5 g were used. They were maintained on Altromin[®] pellets and drinking water in a temperature and humidity controlled room with 12-h light-dark cycles for more than 3 days before use. CTX was injected s.c. in a volume of 2 ml; blood was obtained under light ether anaesthesia by cardiac puncture.

2.3. Investigations in man

2.3.1. Six male healthy volunteers aged 27—32 years, 67—75 kg body weight, were injected i.v. with 1 g CTX in 10 ml sterile aqueous solution within 3 min. Blood samples were collected through an indwelling catheter (Abbotath T Nr. 4535 Abbott, Ltd. Sligo, Ireland) at predetermined times and assayed for CTX and dCTX.

2.3.2. Eight patients, 3 males aged 68—78 years and 48—76 kg body weight, and 5 females aged 58—82 years and 51—89 kg body weight, were treated i.v. with 2 g CTX in aqueous solution. Bile samples were withdrawn through a catheter introduced into the ductus choledochus. Samples were collected at predetermined times and assayed for CTX and dCTX.

2.4. Microbiological assay

2.4.1. Antimicrobial activity

It was assayed by the agar diffusion test. Mueller-Hinton agar and an inoculum of 2×10^6 cfu/ml prepared from fresh overnight culture were used. Plates of 15.4×26.3 cm size were incubated at 37°C for about 18 h. As test organisms *Proteus mirabilis* ATCC 14273, *Pseudomonas aeruginosa* K 1118 and *Escherichia coli* V 6311/65 were used. Inhibition zones were measured with a zone reader. Concentrations of the antimicrobial substance were estimated from the inhibition zone area by comparison with the inhibition zone area from simultaneously determined samples of standard solutions of CTX. The regression line of the logarithmic concentration of CTX against the area of inhibition zone was calculated using a Hewlett-Packard 97 calculator.

2.4.2. Minimal inhibitory concentrations (MIC)

They were determined by serial dilutions of the antibiotics in Mueller-Hinton broth and the MIC was defined as the lowest

concentration of antibiotics that inhibited development of visible growth within 24 h. In addition to the strains mentioned above MIC of CTX and dCTX for *Bacterium cereus* var. *mycoides* were also determined.

2.5. High-performance liquid chromatography (HPLC) assay

2.5.1. Sample preparation

The samples were stored at -70°C and thawed just prior to analysis. An equal volume of 0.4 mol perchloric acid was added to 100—300 μl of serum or bile. The samples were kept at 0°C for 15 min and centrifuged at 10 500 g for 2 min. The clear supernatant was removed and incubated for 10 min at 25°C in order to form dCTXL from dCTX. The solution was buffered with 10% (v/v) 4 mol sodium acetate to a final pH = 4.5. Standard mixtures of CTX : dCTX = 1:1 (w/w) were prepared in serum or bile. Aliquots of 20—25 μl were injected into the chromatographic system.

2.5.2. Chromatographic separation

The chromatographic system consisted of a pump M 6000A, a universal injector U 6 K with a 2-ml loop (an autosampler WISP 710 A for the bile samples), a UV absorbance detector M 440 (254 nm) and a data module M 730, all from Waters Assoc. Separations were achieved with a reversed-phase column, Hibar RT 250-4 LiChrosorb RP 18 7 μm (i.d. 250×4 mm, Fa. Merck, D-6100 Darmstadt). A guard column (i.d. 22×4 mm) dry filled with Corasil C 18 37—50 μm (Waters Assoc.) was used to protect the analytical column. The guard column was replaced after 100—150 injections. For elution a mixture of 20 mmol sodium dihydrogenphosphate in water-methanol-acetonitril (83:7:10; v/v/v) was used. The flow rate was maintained at 1.5 ml/min. The retention time for CTX was about 4.8 and that of dCTXL about 7.5 min.

2.5.3. Quantitation

Concentrations of CTX and dCTX were calculated integrating the peak area with the data module M 730 (Waters Assoc.) using the external standard method. Standard curves of CTX and dCTX in serum gave correlation coefficients of $r > 0.998$ in the range of 0.5—100 $\mu\text{g/ml}$ and 1.25—100 $\mu\text{g/ml}$, respectively (note that dCTX is chromatographed and calculated after conversion into the corresponding lactone). Five aliquots of a mixture of 0.5 $\mu\text{g/ml}$ CTX and 1.25 $\mu\text{g/ml}$ dCTX were prepared as described in 2.5.1. The following chromatography gave a relative standard deviation of the peak area of 10% for CTX and 5% for dCTXL. For routine analyses a single mixture of CTX and dCTX (each 25—50 $\mu\text{g/ml}$) served as external standard. The limit of detection in serum is about 0.5 $\mu\text{g/ml}$ for CTX and 1 $\mu\text{g/ml}$ for dCTX, if analysed as dCTXL as described above. In bile, with a more variable composition, the limit of detection is about 0.8 and 1.5 $\mu\text{g/ml}$, respectively.

3. Results

3.1. Experiments in rats

In 2 groups of 12—15 rats CTX (20 mg/kg) was injected s.c. and serum levels of the compound assayed at the time indicated in Fig. 1. When CTX was determined by bioassay (*P. mirabilis* ATCC 14273) the peak concentration of CTX was obtained about 25 min after drug injection. Serum concentration of CTX declined with half-lives of 31 and 32 min. Assay of CTX by HPLC resulted in considerably lower concentrations especially 150 min after injection of the drug. A shorter half-life of 26 min for CTX was observed.

In addition besides CTX another peak appeared in the chromatogram indicating a metabolite of CTX. Therefore, it seemed likely that the twofold higher concentration of CTX calculated from the antimicrobial activity against *P. mirabilis* was due to an active metabolite of the parent compound.

The chemical structure of CTX and the metabolic fate of other 3-acetoxymethyl cephalosporins [1] led to the confirmative conclusion that the active metabolite of CTX is dCTX indeed. Using authentic dCTX added to the serum of pretreated rats and subsequent analysis by HPLC

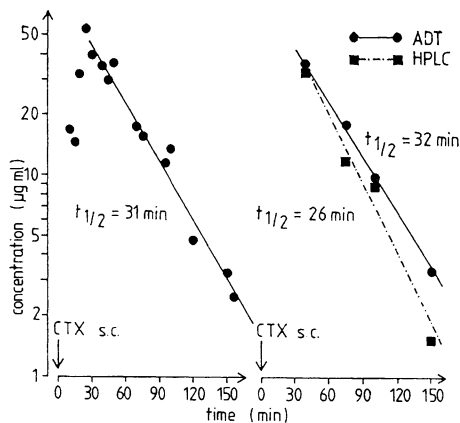


Fig. 1: Serum levels of cefotaxime in 2 groups of rats after s.c. injection of 20 mg/kg determined by HPLC and agar diffusion test (ADT) with the indicator organism *P. mirabilis* ATCC 14273.

showed an increment of the unknown peak with identical shape. By adding perchloric acid to the serum samples (see methods) dCTX is converted to the respective dCTXL, which caused the peak of the metabolite observed in the chromatogram. The rapid formation of lactones from 3-hydroxymethyl cephalosporins by acids is a well known reaction [10].

Thus the described method of HPLC allows the quantitative determination of CTX and its microbiologically active metabolite in biological fluids. However, the non-discoverability of lactone in vivo is a prerequisite for the analytical approach [8]. Both the biological assay and the HPLC were used for a comparative investigation of the concentrations of CTX and dCTX in human serum and bile.

3.2. Studies in healthy volunteers and patients

In Fig. 2 serum concentrations of CTX and dCTX in healthy volunteers obtained by HPLC and microbiological assay procedures, respectively, are depicted. After i.v. injection of 1 g CTX serum samples were collected at the times indicated in Fig. 2. The parent compound was assayed both by microbiological analysis using *P. aeruginosa* K 1118 as test organism and by HPLC. There was an excellent agreement of the concentrations of CTX determined by both methods (correlation coefficient $r = 0.985$; $n = 32$).

From the time-concentration curves elimination half-lives of about 59 min (microbiological assay) and 56 min (HPLC) were estimated. The concentration of dCTX in serum assayed by HPLC increased rapidly, reaching the concentration of CTX about 150 min after the injection and twofold higher concentrations compared with CTX 4 h after drug injection. A half-life of 94 min was estimated

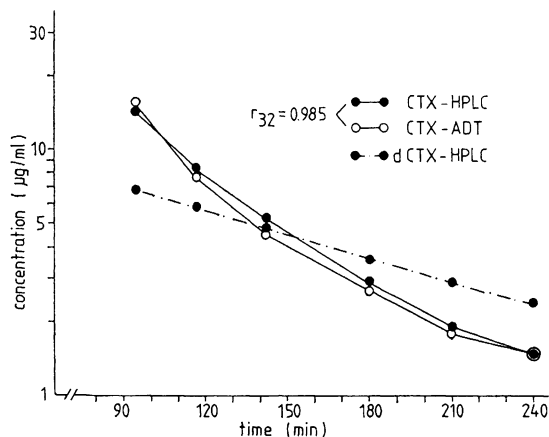


Fig. 2: Mean serum concentrations of cefotaxime and desacetyl cefotaxime in healthy volunteers ($n = 6$) after i.v. injection of 1 g cefotaxime. Comparison of HPLC and microbiological procedure (agar diffusion test — ADT) with the indicator organism *P. aeruginosa* K 1118 (insensitive to desacetyl cefotaxime).

for the metabolite from the time-concentration curve in Fig. 2.

In Table 1 serum concentrations of CTX in a volunteer obtained by microbiological analysis with a strain sensitive against dCTX (*P. mirabilis* ATCC 14273) and an insensitive strain (*P. aeruginosa* K 1118) in comparison to HPLC analysis are shown. It is evident that the serum concentration assayed with the sensitive test organism against the active metabolite resulted in false high values for the parent compound, whereas the serum concentrations of CTX determined with the insensitive test organism and HPLC, respectively, revealed almost identical values ($r = 0.9998$).

In patients receiving 2 g CTX i.v. time-concentration curves of CTX and its desacetyl metabolite were established from samples of bile by HPLC and microbiological assay using *E. coli* V 6311/65 (Fig. 3). The test organism is slightly susceptible to dCTX (Table 2) when compared to *P. aeruginosa* K 1118. Therefore, the concentrations of the parent compound are overestimated by the microbiological procedure. 1.5 h after the injection of the drug the dCTX concentration exceeded the concentration of the parent drug and remained higher than CTX during the time investigated.

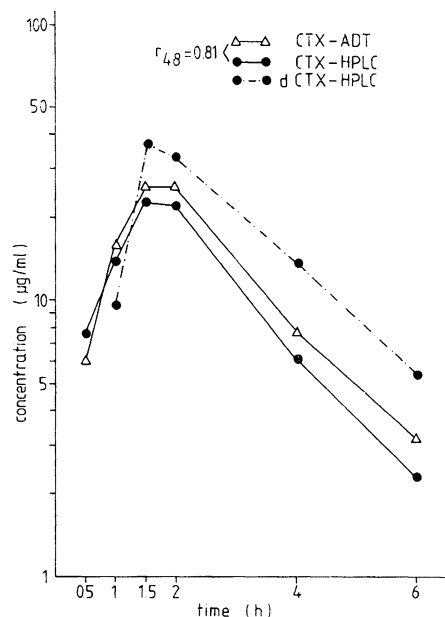


Fig. 3: Mean concentrations of cefotaxime and desacetyl cefotaxime in bile of patients ($n = 8$) after i.v. injection of 2 g cefotaxime. Comparison of HPLC and microbiological procedure (agar diffusion test — ADT) with the indicator organism *E. coli* V 6211/65 (partially sensitive to desacetyl cefotaxime).

Table 1: Mean serum concentrations ($\mu\text{g/ml}$) of cefotaxime (CTX) and desacetyl cefotaxime (dCTX) in a healthy volunteer after i.v. injection of 1 g cefotaxime. Comparison of HPLC method with microbiological assay procedures (agar diffusion test — ADT). *P. mirabilis* ATCC 14273 — sensitive to dCTX; *P. aeruginosa* K 1118 — insensitive to dCTX.

Estimated compound method used	Time after injection (min)				
	75	95	117	142	180
CTX-ADT- <i>P. mir.</i>	28.9	17.8	9.3	7.5	5.6
CTX-ADT- <i>P. aerug.</i>	26.9	10.3	7.4	5.6	3.7
CTX-HPLC	29.3	10.6	7.9	6.0	3.6
dCTX-HPLC	7.6	6.5	5.8	4.4	3.2

Tab. 2: Minimum inhibitory concentrations (MIC) of cefotaxime (CTX) and desacetyl cefotaxime (dCTX) against different strains.

	<i>B. cereus</i> var. <i>mycoides</i>	<i>P. aeruginosa</i> K 1118	<i>E. coli</i> V 6311/65	<i>P. mirabilis</i> ATCC 14273
CTX	0.025	0.4	0.013	0.025
dCTX	0.025	20	0.125	0.2

4. Discussion

During the last five years new semisynthetic cephalosporins have been developed which possess broad-spectrum antibacterial activity especially against gram-negative organisms and also have an enhanced stability to various classes of β -lactamases [2].

Among these compounds, CTX has been shown to be a very active cephalosporin with a high affinity to the binding proteins [5] and the ability to penetrate into bacterial cells such as *Enterobacter cloacae* [6]. Therefore, the pharmacodynamic and pharmacokinetic properties of CTX are very important for rational use in the therapy of serious infections. Usually serum concentrations of antibiotics are estimated by the agar diffusion method using sensitive test organisms. This method can be of limited value when the parent compound is degraded to an active metabolite, and the strain of bacteria used for the bioassay is sensitive to this active metabolite.

In the present paper the occurrence of an active metabolite of CTX has been demonstrated both by bioassay and high-performance liquid chromatography. The first attempt to determine CTX by bioassay with the test organism *P. mirabilis* resulted in spuriously high concentrations of the cephalosporin in serum. *P. mirabilis* was selected for the test because we used this micro-organism for experimental infections in rats, and subsequent treatment with CTX was performed. It could be shown that *P. mirabilis* was sensitive both to the parent compound and to its active metabolite dCTX (Table 2).

The use of *P. aeruginosa* K 1118 as test organism, which is insensitive to dCTX at concentrations below 100 $\mu\text{g/ml}$ (Table 2), resulted in a highly significant correlation between the concentrations of CTX found in human serum of volunteers assayed simultaneously by the agar diffusion method and HPLC ($r = 0.985$; Fig. 2; Table 1). From the concentrations of CTX and dCTX in human serum assayed by HPLC a half-life of 56 min for the parent compound and 94 min for dCTX was obtained. Because of the prolonged elimination of the active metabolite it is necessary to estimate serum concentrations of CTX in the late elimination phase either by HPLC or a test organism insensitive to dCTX.

CTX in serum samples at room temperature showed a measurable degradation of the compound to dCTX, which could be avoided at 4°C for a short time. For storage of samples over a long period (several months), a temperature of -70°C is necessary.

In agreement with previous results [9] we found also a rapid and quantitative desacetylation of CTX in serum containing haemolysed erythrocytes. The presence of CTX and dCTX in bile of patients demonstrated by HPLC and bioassay indicates the metabolic formation of degradation products of CTX in the liver. In experiments in rats with impaired liver function, only very small amounts of the desacetyl metabolite in serum after injection of the 3-acetoxymethyl compound cephalothin were found [4].

Our results point to the importance of specific microbiological assays or analytical procedures like HPLC for the precise determination of pharmacokinetic parameters of 3-acetoxymethyl cephalosporins.

5. References

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