Supporting Information for article:

Targeted Hydrolysis of β-Lactam Antibiotics in Dry Suspension Remains: A Proposed Method to Reduce Ecological Toxicity and Bacterial Resistances

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Content of SI

1) Material & Methods, sample preparation

2) HPLC analysis of amoxicillin, cefaclor, cefadroxil, cefuroxime

3) Validation parameters

1) Material & Methods

All chemical reagents were commercially available and were used without further purification from abcr GmbH, Karlsruhe, Germany; Sigma-Aldrich Chemie GmbH, Merck Group, Munich, Germany; Merck Millipore, Darmstadt, Germany; Acros Organics, Germany.

chemicals	manufacturer	purity/concentration
acetonitril	Honeywell	99.99 %
acetic acid	Baker	99-100 %
acetic acid (LC MS)	Roth	100 %
ammonium acetate	TCI	97 %
ammonium acetate (LC MS)	Merck	>98
amoxicillin	Acros Organics	96 %
amoxicillin dry suspension	Aluid Pharma	250 mg/ml
cefaclor	Acros Organics	96 %
cefaclor dry suspension	Aristo	250 mg/ml
cefadroxil	abcr	95 %
cefadroxil dry suspension	Infecto PHARM	500 mg/ml
cefuroxime	abcr	95 %
cefuroxime dry suspension	SUN Pharma	125 mg/ml
methanol	Baker	Hplc grade
sodium hydroxide	Walter CMP	98 %
sodium hydroxide	Alfar Aesar	99.99 %
paracetamol	Rhodia Organics	n.a
tetramethylammoniumchloride	ROTH	98 %

All prepared dry suspension formulations were stored at 8°C and all probes were measured within three weeks after preparation. All samples of the pure compounds were freshly prepared and immediately used for measurements.

All HPLC measurements were performed using a Waters 600 E HPLC System coupled with a Waters 717 plus autosampler over a Waters X Bridge C18 5 µm 4.6mmx250mm column with a Vanguard X Bridge BEH C18 5 µm precolumn. The mobile phase was previously degassed with a Knaur Degasser. The detector was set to a wavelength of 254 nm for amoxicillin and cefaclor, and was set to 280 nm for cefadroxil and cefuroxime.

LC/MS was performed using an Agilent 1100 HPLC system and an Agilent Eclipse XDB-C8 column. Mass spectra were recorded on a connected Bruker Esquire ~LC ion trap or amaZon SL ESI mass spectrometer.

Preparation of sample solutions (n = 3)

Due of the varying API concentrations in dry suspension formulations and their respective spectroscopic properties it was necessary to adapt the preparation of the HPLC samples. Paracetamol was used as internal standard for all analytical samples. Reference compounds were used for API identification in samples of prepared suspension formulations:

amoxicillin Across Organics, purity 96%
cefaclor Across Organics, purity 96%
cefadroxil abcr purity 95%
cefuroxime abcr purity 95%

compound	pdsf*: NaOH [V:V]	NaOH conc. [n]	time of quantitative hydrolysis [min]
amoxicillin	1:1	0.5	20
cefaclor	1:1	0.4	20
cefadroxil	1:2	1.0	60
cefuroxime	1:2	1.0	240

*prepared dry suspension formulation

The dilution solution of **amoxicillin** was prepared from 20.0 mg paracetamol and 2.5 ml acetic acid in 197.5 ml filtered distilled water.

For diluting of the **cefaclor** probes a solution of 20.0 mg paracetamol and 308 mg ammonium acetate was diluted in 4 ml acetonitrile and 169 ml filtered bidest water. By adding acetic acid dropwise to the solution a pH value of 4 was obtained.

For **cefadroxil** and **cefuroxime** the same dilution solution was used. It consisted out of 20.0 mg paracetamol and 308 mg ammonium acetate in 10 ml acetonitrile and 190 ml filtered distilled water. The pH was adjusted to 5 by adding acetic acid.

Preparation of HPLC samples

The **amoxicillin** and **cefaclor** probes were prepared by adding 1.0 ml of the alkaline dry suspension reaction mixture to 9.0 ml of the dilution solution. After mixing the solution was filtered through a LLG filter.

The **cefadroxil** and **cefuroxime** probes were prepared by adding 0.2 ml of the alkaline dry suspension reaction mixture to 10.0 ml of the dilution solution. The probe was then mixed and filtered through a LLG filter.

2) HPLC analysis

Amoxicillin HPLC method

The mobile phase for determination of **amoxicillin** consisted of methanol (eluent A) and eluent B (Table S1) which was prepared by adding 25 ml acetic acid (100 %) and 100 ml methanol in a cylinder which was then filled up to the 2 L benchmark with filtered distilled water. Then 2.065 ml of a 10 M sodium hydroxide were added to the eluent to adjust the pH value = 3.5. A gradient was used to separate amoxicillin from its degradation products (Table S1).

Table S1. HPLC gradient of mobile phase for the amoxicillin method.

time	Flow rate (ml/min)	A (MeOH)	B (5% MeOH in 1.25 % AcOH)
0	1.5	0	100
8.5	1.5	0	100
10	1.2	15	85
15	1.2	15	85
17.5	1.2	5	95
20	1.2	0	100
30	1.5	0	100

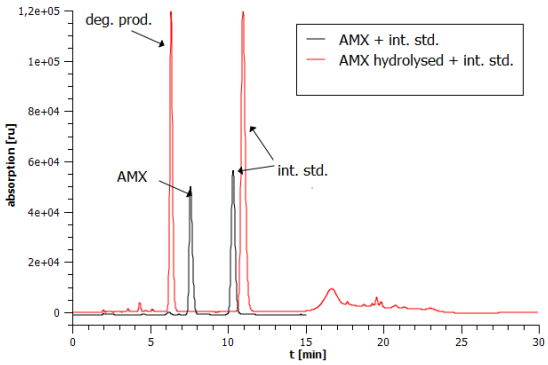
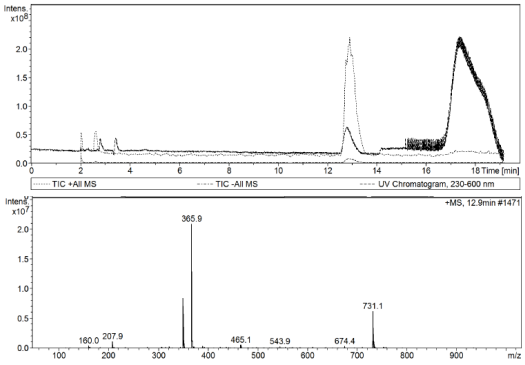
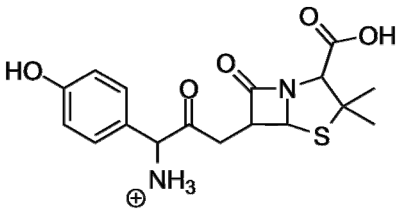


Figure S1. HPLC chromatogram of a freshly prepared suspension form *Amoxicillin Aluid Pharma* 250mg/5mL in **black**, rt amoxicillin AMX = 7.6 min, rt paracetamol as int. std. = 10.4 min (flow rate 1.5 ml/min, 5% MeOH in 1.25 % AcOH).
Chromatogram of a hydrolyzed sample in **red**, rt degradation product penicillo acid derivative of AMX (deg. prod.) = 6.4 min, rt int. std = 11.1 min. Retention time of the int. std. varies because the hydrolyzed probes were measured with gradient conditions (see method).

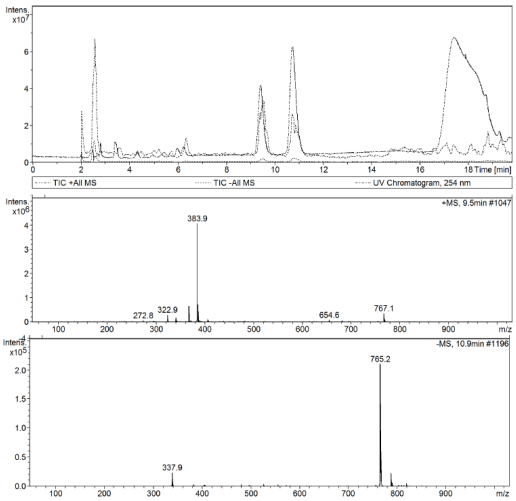
A

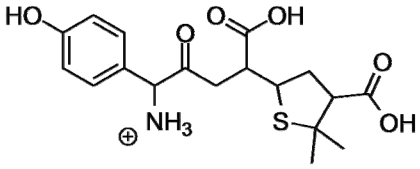




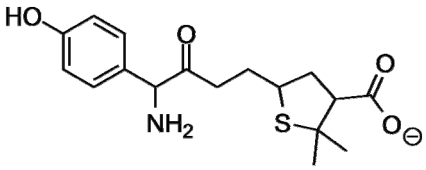
rt 12.9 min: amoxicillin (m/z = 365.9)

B





rt 9.5 min: penicillo acid derivate of amoxicillin (m/z = 383.9)



rt 10.9 min: penillo acid derivate of amoxicillin (m/z = 337.9)

Figure S2 A LC/MS analysis of amoxicillin from freshly prepared suspension formulation *Amoxicillin Aluid Pharma* 250mg/5ml (rt amoxicillin = 12.9 min; m = 365.1). **B** Sample of the mixture outlined in **A** quantitatively hydrolyzed within 20 min by 0.5 M NaOH yielding a mixture of products (main peaks: rt = 9.5 min; rt = 10.9 min; rt = 12.9 min).

Cefaclor HPLC method

For the **cefaclor** method eluent A was acetonitrile and eluent B (Table S2) was prepared by adding 3.08 g of ammonium acetate to a solution of 100 ml acetonitrile and 1900 ml of filtered distilled water. The pH value was adjusted to 4 by carefully adding acetic acid. The chromatographic conditions to separate cefaclor from its degradation products are shown in Table S2.

Table S2. HPLC gradient of mobile phase for the cefaclor method.

time	Flow rate (ml/min)	A (ACN)	B (20 mmol NH_4AcO in 5 % ACN pH=4)
0	1	0	100
30	1	13	87
35	1	48	52
40	1	0	100
60	1	0	100

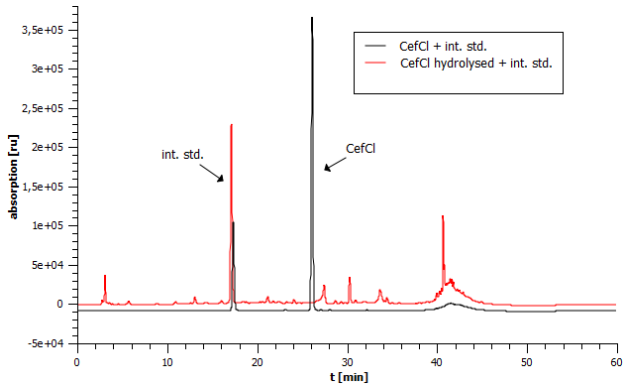
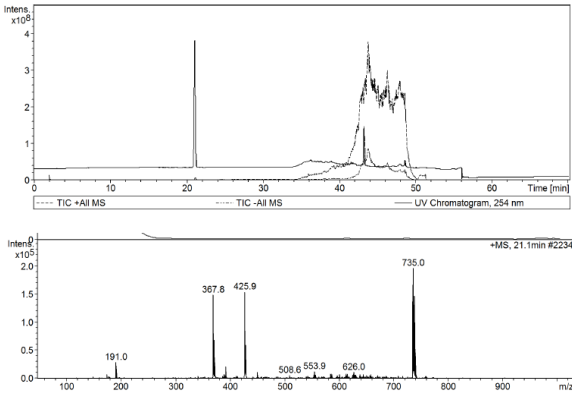
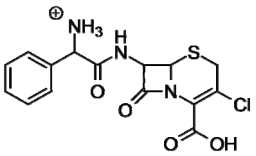


Figure S3: HPLC chromatogram of a freshly prepared suspension form *Cefaclor Aristo* 250mg/5ml (black, rt cefaclor = 26.1 min, rt int. std. = 17.5 min). Chromatogram of a hydrolyzed sample (red, int. std = 17.0 min).

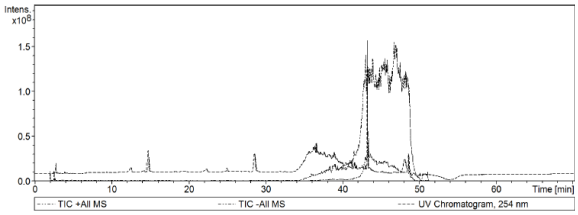
A

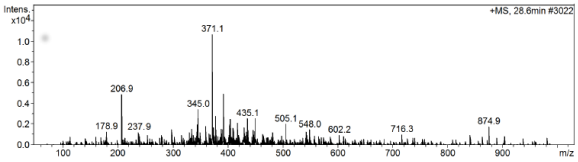


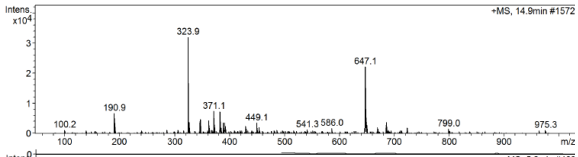


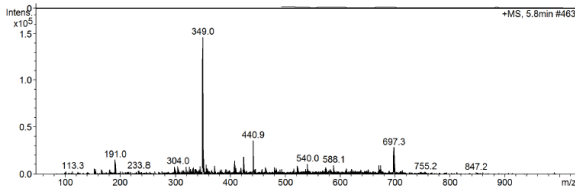
rt 21.1 min: cefaclor (m/z = 367.8)

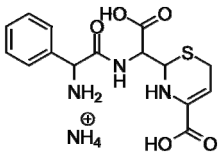
B



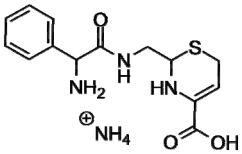




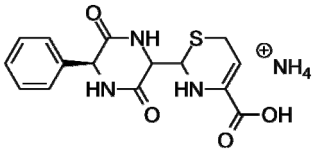




rt 28.6 min: penicillo acid derivate of cefaclor (m/z = 371.1)



rt 14.9 min: penillo acid derivate of cefaclor (m/z = 323.9)



rt 5.8 min: diketopiperazine derivative of cefaclor (m/z = 349.0)

Figure S4 **A** LC/ MS analysis of cefaclor from freshly prepared suspension formulation *Cefaclor Aristo* 250mg/5ml (rt cefaclor = 21.1 min). **B** Sample of the mixture outlined in **A** quantitatively hydrolyzed within 20 min by 0.4 M NaOH yielding a mixture of products (main peaks: rt = 5.8 min; rt = 14.9 min; rt = 21.1 min).

Cefadroxil HPLC method

As eluent A for the **cefadroxil** method a 20 mmol ammonium acetate solution with 5 % acetonitrile was used (Table S3). For preparation, 3.08 g ammonium acetate was diluted in a mixture of 100 ml acetonitrile and 1900 ml filtered distilled water. The pH was then adjusted to pH 4 by adding acetic acid. The eluent B (Table S3) used for the cefadroxil method was identical with the eluent from the cefaclor method (eluent B, Table S2). The gradient used to separate cefadroxil from its degradation products is given in Table S3.

Table S3. HPLC gradient of mobile phase for the cefadroxil method.

time	Flow rate (ml/min)	A (20 mmol 20 mmol NH_4AcO in 2 % ACN pH=4)	B (20 mmol NH_4AcO in 5 % ACN pH=4)
0	1	100	0
20	1	75	25
20.01	1.3	75	25
30	1.3	100	0
35	1	100	0

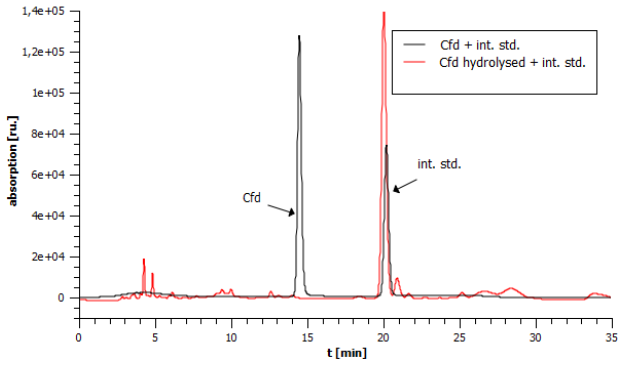


Figure S5: HPLC chromatogram of a freshly prepared suspension from *Cefadroxil InfectoPharm* 500mg/5ml (black, rt cefadroxil = 14.5 min, rt int. std. = 20.2 min). Chromatogram of a hydrolyzed sample (red, int. std = 20.2 min).

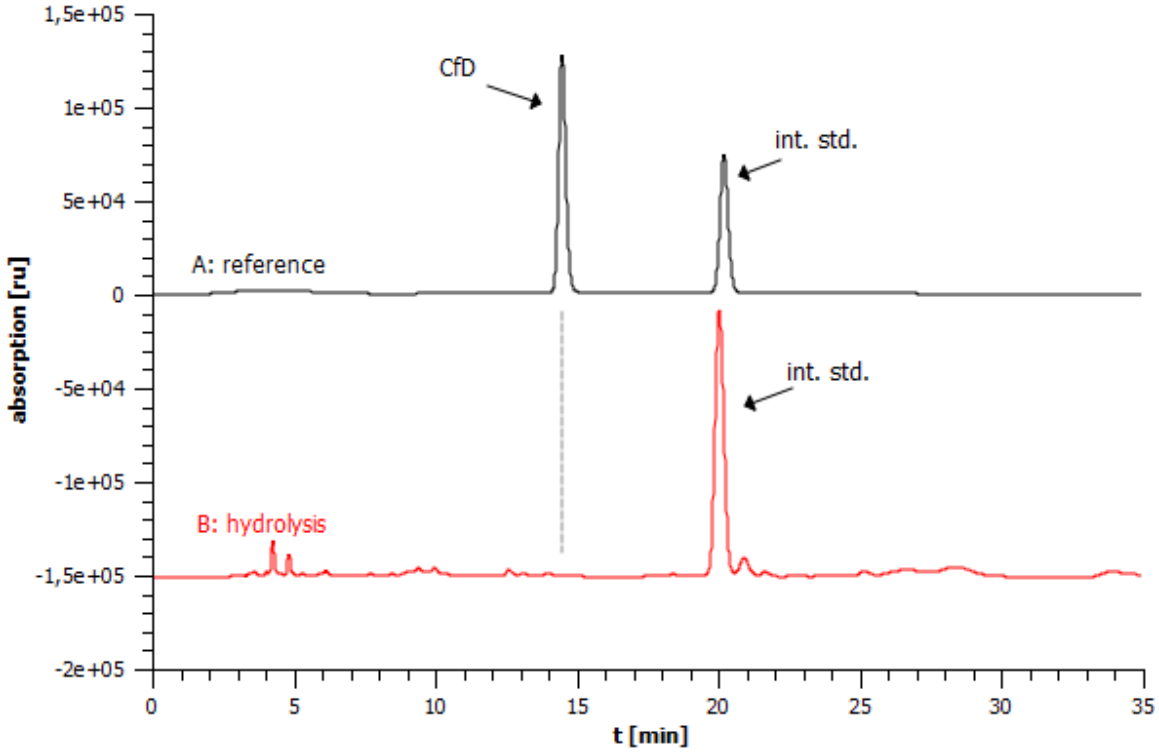
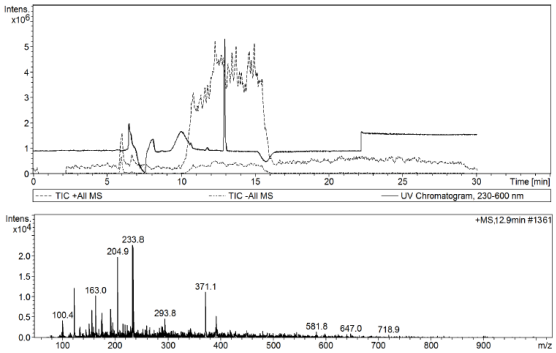
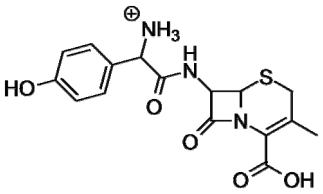


Figure S6 A. Reference HPLC chromatogram of prepared dry suspension of cefadroxil (CfD) with paracetamol as internal standard (int.std.). Retention times rt CfD = 14.5 min, rt int. std. = 20.3 min. **B**. A sample mixture of CfD and int.std. was stirred for 60 min with twice the volume of 1.0 M NaOH, which quantitatively hydrolyzed CfD. LC/MS analysis of the degradation products can be found in **Figure S7**.

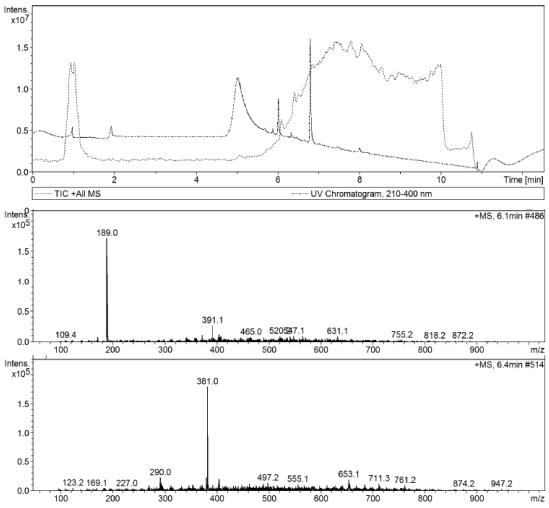
A

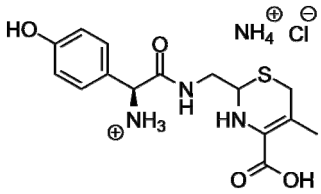




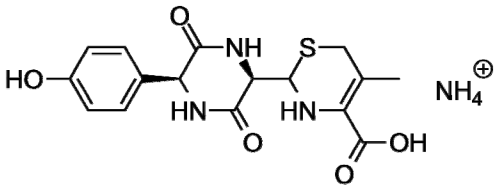
rt 13 min: cefadroxil

B





rt 6.1 min: penillo acid derivate of cefadroxil (m/z = 391.1)



rt.6.4 min: diketopiperazine derivate of cefadroxil (m/z = 381.0)

Figure S7 A LC/MS analysis of cefadroxil from freshly prepared suspension formulation *Cefadroxil InfectoPharm* 500mg/5ml (rt cefadroxil = 21.3 min). **B** Sample of the mixture outlined in **A** quantitatively within 60 min hydrolyzed by 1M NaOH yielding a mixture of products (main peaks: rt = 5.1 min; rt = 6.4 min).

Cefuroxime HPLC method

Eluent A for the **cefuroxime** method was acetonitrile and eluent B (Table S4) was identical with eluent B of the cefadroxil method (Table S3). The chromatographic conditions for cefuroxime are given in Table S4.

Table S4. HPLC gradient of mobile phase for the cefuroxime method.

time	Flow rate (ml/min)	A (ACN)	B (20 mmol NH_4AcO in 5 % ACN pH=4)
0	0.4	15	85
12	0.4	30	70
29	1	15	85
30	0.4	15	85

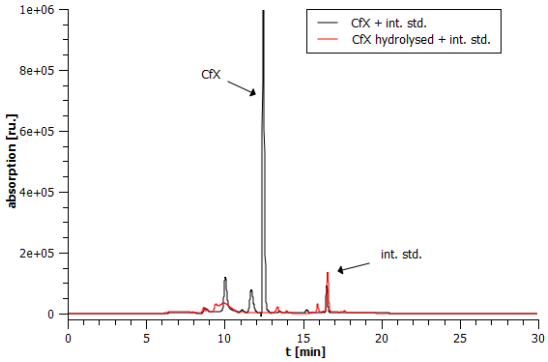
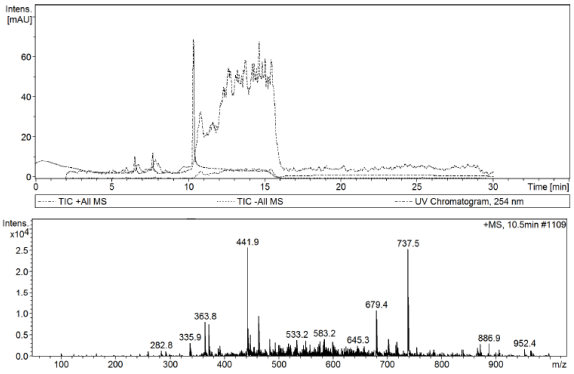
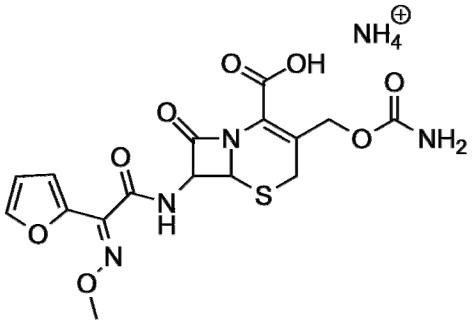


Figure S8: HPLC chromatogram of a freshly prepared suspension from *Cefuroxim InfectoPharm* 500mg/5ml (black, rt cefuroxime = 12.5 min, rt int. std. = 16.5 min). Chromatogram of a hydrolyzed sample (red, int. std = 16.5 min).

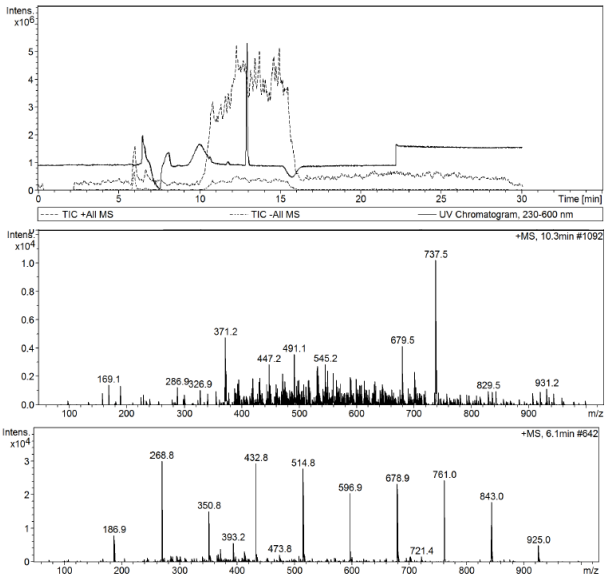
A

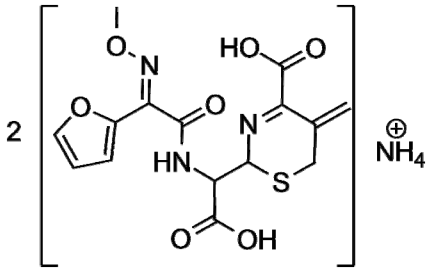




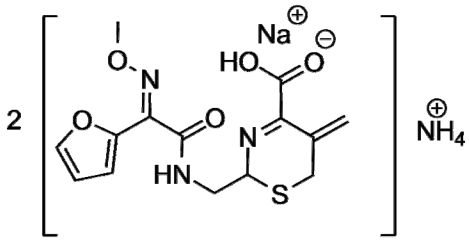
rt 10.5 min: cefuroxime (m/z = 441.9)

B





rt 6.1 min: penicillo acid derivate of cefuroxime (m/z = 678.9) peak ca 13 min??



rt 10.3 min: penillo acid derivate of cefuroxime (m/z = 737.5)

Figure S9 A. LC/MS analysis of cefuroxime from freshly prepared suspension formulation *Cefuroxime SUNPharma* 125mg/5ml (rt cefuroxime = 10.5 min). **B.** Sample of the mixture outlined in **A** quantitatively hydrolyzed by 1M NaOH yielding a mixture of products within 240 min (main peaks: rt = 6.1 min; rt = 10.3 min).

3) Validation parameters

The analytical performance of the respective method was checked for specificity, limit of quantification, limit of detection, trueness, precision and robustness.

The **specificity** of the chromatographic method was characterized using the resolution *R* (1), the symmetry factor *T* (2) and the theoretical plate number *N* (3).

resolution: $$R = \frac{2(t_{r2} - t_{r1})}{w_1 - w_2} \quad (1)$$

symmetry factor: $$T = \frac{a + b}{2a} \quad (2)$$

theoretical plate number: $$N = 16\left(\frac{t_r}{w}\right)^2 \quad (3)$$

abbreviations

t_{r1} = retention time peak 1
t_{r2} = retention time peak 2
a = peakwith befor peakmax.
b = peakwith after peakmax.
w_1 = peakwith from peak 1
w_2 = peakwith from peak 2
t_r = retention time
w = peakwith

The **limit of quantification** for each compound to be analyzed was determined by identifying the concentration at which the variation coefficient of a triple determination is equal to 10. The **limit of detection** equals one third of the limit of quantification.

The **trueness** was verified with calibration solutions of compounds, respectively. Three probes were prepared as shown in Table S5 and then the peak areas of the HPLC chromatograms were analyzed by formula (4).

Table S5. Parameters for the determination of trueness.

	probe solution	calibration solution	solvent
S1	1	1	0
S2	1	0	1
S3	0	1	1

$$\text{recovery: } W = \frac{S1 - S2}{S3} \cdot 100 \quad (4)$$

The **precision** of the analytical method was determined by repeating the sample analysis six times at two different days and calculating the variation coefficients.

The **robustness** of the analytical method was tested by hydrolyzing the dry suspension probe with only 75 % of the sodium hydroxide volume, 75 % of the sodium hydroxide concentration and 75 % of the maximal reaction time, respectively.