J. Pharm. Pharmacol. 1982, 34: 425–428
Received October 13, 1981

0022–3573/82/070425–04 $02.50/0

A stability-indicating assay for cefotaxime† utilizing thin-layer chromatography with fluorescence detection

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A t.l.c. method on silicagel plate with a fluorescence indicator has been carried out to increase the native fluorescence of cefotaxime and four of its degradation products. A simultaneous improved fluorimetric quantitation of the compounds in situ is proposed using a chromatogram spectrophotometer. The detectability (0·20 μg) shows that the procedure is twenty times more sensitive than high-pressure liquid chromatography with u.v. detection; it allows the determination of traces level (1%) degradation products.

The establishment of sensitive methods of separation and quantitation of decomposition products of cephalosporins is of particular interest as it has been suggested (Schwartz 1969; Yamana & Tsuji 1976) that these compounds may cause allergic reactions with antibiotics of the β-lactam family. Chromatographic methods quoted for the separation and the identification of cefotaxime(I) and its metabolites refer to u.v. absorption as a detection procedure (White et al 1979; Chamberlain et al 1980; Reeves et al 1980), and high-performance liquid chromatography (h.p.l.c.) with detection at 235 or 254 nm or thin-layer chromatography (t.l.c.) with fluorescence quenching at 254 nm. There are no published references to the fluorescence of cefotaxime and its potential degradation products in vitro (Fig. 1): desacetylcefotaxime (II), desacetoxylcefotaxime (III), desacetylcefotaxime lactone (IV), thiazoximic acid (V), 7-aminocephalosporanic acid (VI). In this paper, a t.l.c. method on silicagel plates with a fluorescence indicator and fluorimetric detection at 315 nm is proposed, which allows the quantitation of trace levels of (I), (II), (III), (IV) and (V) using a chromatogram spectrophotometer. This method permits the simultaneous determination of each degradation product to monitor the stability of (I).

MATERIALS AND METHODS

Apparatus

A chromatogram spectrophotodensitometer (Zeiss PMQ II) with a chart recorder and an electronic integrator was used. The radiation source was deuterium lamp or mercury vapour lamp.

Material and reagents

Precoated silicagel 60 and silicagel 60 F_{254} plates supplied by Merck (Darmstadt, West Germany), 0·25 mm thickness were used. (I), (II), (III), (IV), (V) and (VI) were gifts from Roussel Laboratories and were used as received. Methanol, ethylacetate, acetone were distilled before use, acetic acid was of analytical grade and doubly distilled water was used. The mobile phase was ethyl acetate–acetone–water–acetic acid (50:25:15:10% v/v).

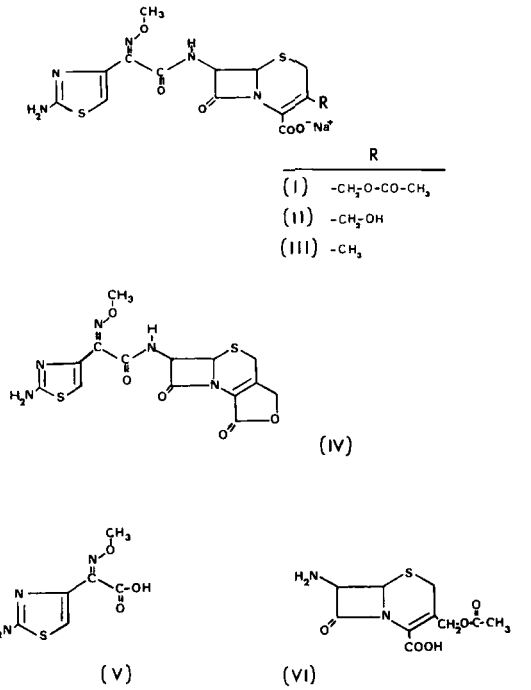

FIG. 1. Structure of cefotaxime and potential degradation compounds.

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Standard solutions: a mixed standard stock solution of (I), (II), (III), (IV) and (V) (0·2 mg ml^{-1} each, was prepared in methanol. This primary solution was diluted with methanol before use to give solutions in the concentration range of 0·005 to 0·025 mg ml^{-1}.

Test solutions: cefotaxime methanolic solutions were 0·430 mg ml^{-1} with added amounts (1% with respect to (I)) of (II), (III), (IV) and (V) and 0·430 mg ml^{-1} with added amounts (3% with respect to (I)) of (II), (III), (IV) and (V).

Chromatography

A 3 µl loading of each standard solution was applied to the t.l.c. plate (10 µl Hamilton syringe). The chromatogram was allowed to develop to a height of about 16 cm in a tank previously saturated with the mobile phase, and was dried under a stream of cold air.

Qualitative and quantitative analysis of each spot was performed either by reflectometric measurements at 270 nm (maximum absorbance of (I), (II), (III), (IV) and (V) on the plate) or by fluorescence emission using a mercury vapour lamp with an excitation wavelength of 315 nm (maximum excitation wavelength of each compound) and a filter with a cut off at 390 nm. The scanning speed of the paper was fixed at 60 mm min^{-1} and the scanning speed of the plate was 50 mm min^{-1}.

RESULTS AND DISCUSSION

Fluorescence of (I), (II), (III), (IV), (V), (VI) in the solid state was examined as a qualitative test under a u.v. lamp at two excitation wavelengths: 254 and 350 nm. The results are presented in Table 1.

A separation of (I), (II), (III), (IV), (V) and (VI) was carried out by t.l.c. The spots were located using u.v detection (270 nm). The respective R_F values are in Table 1. The results show the apparent resolution between (V) and (VI) obtained on a plate with a fluorescence indicator is better than on a plate without one.

Fluorescence

Loadings of a mixed standard solution (75 ng of each compound) were applied to a silicagel 60 plate; compounds (I), (II), (III) were fluorescent and compounds (IV), (V) and (VI) were not detected; with the same loading on a silicagel 60 F_{254} plate, a dramatic increase in fluorescence for (I), (II), (III) and a noticeable fluorescence was observed for (IV) and (V) using the same excitation wavelength (315 nm). The influence of pH was investigated by spraying the t.l.c. plates with several reagents. An inhibition of fluorescence with sulphuric acid (10% v/v in ethanol), a diminution of fluorescence with glycocolle buffer (pH = 9·5 and pH = 10·5) and an unchanged fluorescence with citric acid-sodium citrate buffer (pH = 4·0) were noted both on silicagel 60 and silicagel 60 F_{254} plates.

Because of the resolution and the sensitivity of the fluorescence, silicagel 60 F_{254} plates were used for further studies and determinations. A chromatogram of a standard solution with fluorescence detection on a silicagel 60 F_{254} plate is given in Fig. 2.

The stability of the fluorescence emission was tested by scanning the plates four times using the chromatogram spectrophotodensitometer. No diminution of the fluorescence was observed within the limits of repeatability of the instrument (≈3%) but a continuous irradiation of the spots for 1 min produced a significant decrease of the fluorescence intensity.

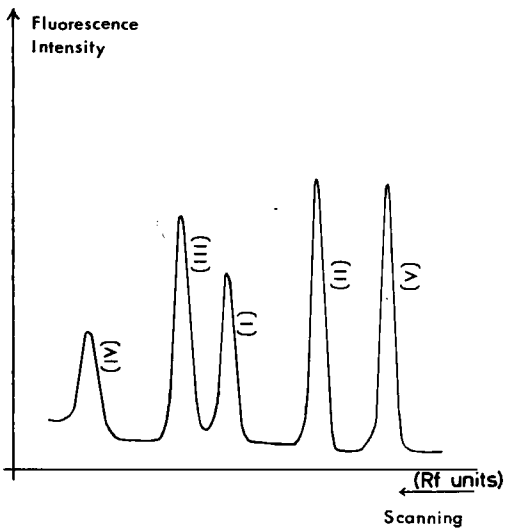

FIG. 2. Chromatogram of a standard solution on a t.l.c. plate with a fluorescence indicator. (I) 25 µg ml^{-1}, (II) 25 µg ml^{-1}, (III) 29 µg ml^{-1}, (IV) 27 µg ml^{-1}, (V) 22 µg ml^{-1}, Loadings: 3 µl, λ exc = 315 nm, λ emission ≥ 390 nm, chart speed: 50 mm min^{-1}, scanning speed: 60 mm min^{-1}, attenuation 100 mV.

Resolution was calculated using the formula:

$$R = \frac{2(d_2 - d_1)}{w_1 + w_2}$$

where $(d_2 - d_1)$ is the distance between the peaks of the components measured at the peak maximum,

Table 1. Fluorescence and R_F values of cefotaxime and its degradation compounds.

Compound	Raw material in solid state: Fluorescence excitation at 254 nm	Raw material in solid state: Fluorescence excitation at 350 nm	Relative to t.l.c. separation: R_F on silicagel 60 plate	Relative to t.l.c. separation: R_F on silicagel 60 F_{254} plate
(I)	Not detected	Bright ++	$0{\cdot}43_8$	$0{\cdot}42_1$
(II)	Yellow ++	Bright ++++	$0{\cdot}26_7$	$0{\cdot}23_4$
(III)	Not detected	Yellow +	$0{\cdot}54_2$	$0{\cdot}52_4$
(IV)	Yellow ++	Yellow ++	$0{\cdot}79_7$	$0{\cdot}74_6$
(V)	Not detected	Dark yellow, very weak	$0{\cdot}18_6$	$0{\cdot}09_6$
(VI)*	Not detected	Dark yellow, very weak	$0{\cdot}18_2$	$0{\cdot}17_0$

* R_F values from alkaline soln in methanol.

and w_1 and w_2 peak widths at the baseline, was always better than 1·8.

The peak skew was evaluated by the *asymmetry coefficient* (Kirkland et al 1976) A_s = b/a where b is the distance after the peak maximum and a the distance before the peak maximum with both a and b, measured at 10% of the total peak height (with the same scanning speed for the plate and the paper) was always ≈1.

The resolution and the asymmetry coefficients found should allow a quantitative analysis of (I), (II), (III), (IV) and (V).

Linearity. The calibration graphs plotted from mixed standard solutions with equivalent concentrations of each compound were linear from 0·005 to 0·025 mg ml^{-1}. The calibration data were generated from five concentrations for each compound. Linear regression equations, confidence limits on the slope and on the intercept (P = 95%) and correlation coefficients from the calibration data were derived for (I), (II), (III), (IV) and (V). The correlation is highly significant (<0·1% level of significance). F tests calculated to check the linearity of the graphs give a linear regression (<0·1% level of significance) for each compound and confirm the calculations of the correlation coefficients (all better than 0·994). The confidence limits on the intercept showed each calibration graph to go through the origin.

Repeatability. The repeatability was checked by applying ten loadings (3 µl for each) of the mixed standard solution (0·015 mg ml^{-1}) of (I), (II), (III), (IV) and (V) on each of five t.l.c. plates. The average assay value for each component was calculated for each of the five plates and the repeatability expressed as the coefficient of variation: 6·44% (I), 4·92% (II), 5·42% (III), 11·95% (IV), 5·27% (V).

Recovery studies. A solution of cefotaxime (0·43 mg ml^{-1}) was spiked with 3% (with respect to (I), and 1% (with respect to (I)) of (II), (III), (IV) and (V). The percentage recovered (average of two determinations) was found to be 96·3% (II), 97·3% (III), 132·8% (IV), 103·6% (V) in the former case, 92·0% (II), 93·0% (III), 131·3% (IV), 106·0% (V) in the later case. Applying the same procedure to the determination of (I) using a suitable dilution, the recovery was 95·73% and 96·5% respectively.

The sensitivity (defined as the change in measured area value resulting from concentration change of one unit) was 210 cm^2 g^{-1} litre^{-1} for (I), (IV), 300 cm^2 g^{-1} litre^{-1} (III), 380 cm^2 g^{-1} litre^{-1} for (II) and 410 cm^2 g^{-1} litre^{-1} for (V) with 50 mV attenuation.

The limit of detection (defined as the amount which gives a height response equal to twice the background) was calculated for (I), (II), (III), (IV) and (V). The results for detectability using as a detection procedure, u.v. absorption (λ = 270 nm), fluorescence quenching (λ =254 nm), and fluorimetric emission (with fluorescence excitation at λ = 315 nm) on t.l.c. plates with and without indicator are compared in Table 2.

Table 2. Comparative results of detectability (µg × 10^3) of cefotaxime and its degradation products from t.l.c. and h.p.l.c. procedures.

	T.l.c.					
	u.v. absorption (λ = 270 nm)			Fluorescence emission		H.p.l.c. (λ = 235 nm)
Compound	−ind*	+Ind**	Fluorescence quenching	−Ind*	+Ind**	
(I)	10	10	20	4	0·25	8
(II)	11	11	20	4	0·20	5
(III)	10	10	20	4	0·25	5
(IV)	20	20	40	not detected with	0·35	8
(V)	6	6	15	75 × 10^{-3} µg	0·15	4

* − Ind: silicagel 60 plate.
** + Ind: silicagel 60 F_{254} plate.

The fluorimetric determination of penicillins and cephalosporins themselves is often based on the formation of degradation products which are fluorescent in solution (Barbhaiya et al 1978), or on the detection of the fluorophores formed after

derivatization with fluorescamine (Crombez et al 1979). The fluorescence observed with (I), (II), (III) and (IV), in solid state, is noticeable but may be affected by the presence of impurities; this drawback is overcome after t.l.c. separation.

The significant increase of fluorescence with excitation at 315 nm we noticed using a plate with a fluorescence indicator has not been observed to our knowledge. The mechanism for this increase may be attributed to a complex with metal ions present in the silicagel coating and/or with the fluorescence indicator.

If this formation is involved it may be rapid and quantitative for (I), (II), (III), (IV) and (V) upon spotting onto the surface of the silicagel as shown by the asymmetry coefficients and the correlation coefficients. A slower and non-quantitative complexation reaction would produce tailing peaks and lower correlation coefficients.

Recovery experiments for the compounds (I), (II), (III) and (V) are satisfactory. For (IV) the recovery of ≈132% together with the coefficient of variation of 11·95% obtained with compound (IV) is believed to result from the fact that compound (IV) migrates at the solvent front with consequent drifting of the baseline.

The main advantage of the proposed procedure on plates with a fluorescence indicator is the limit of detection compared with methods using u.v. absorption, fluorescence quenching or fluorescence emission on plate without fluorescence indicator.

We compared the detectability of this t.l.c. procedure with h.p.l.c. method. H.p.l.c. was carried out on a Spectra-Physics SP 8000 equipped with a variable wavelength detector Schoeffel model SF 770, a 10 µl automatic loop injection, a reversed-phase column 10 µm R.P. 18, 25 cm length, 4 mm diameter, home-made column. The mobile phase was 0·0044 M potassium monobasic phosphate, 0·02 M sodium dibasic phosphate pH = 7·6–methanol (83:17), the flow rate was 1·5 ml min^{-1} and the measurements were recorded at λ = 235 nm, maximum absorbance of (I), (II), (III), (IV) and (V) in the mobile phase. Comparative results of detectability using t.l.c. and h.p.l.c. are given in Table 1.

Conclusion

The proposed procedure enables qualitative and quantitative analysis for the evaluation of the purity of cefotaxime. The same procedure could also be used to monitor the stability of cefotaxime by determination of its degradation products. The specificity of this fluorescence detection procedure which avoids the interference of u.v. absorbing substances and the detectability of this method (20 times more than h.p.l.c.) suggest an application to the biological fluids.

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