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Evaluation of analytical methods: Liquid chromatography of cefalexin

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Summary

A comparative study of three isocratic liquid chromatographic methods for the analysis of cefalexin is described. Two methods, taken from the European Pharmacopoeia and the US Pharmacopeia, use classical alkyl bonded phases (C_{18}) as the stationary phase. The third method uses poly(styrene-divinylbenzene). Poor reproducibility of the selectivity towards cefalexin and its related substances was observed for the C_{18} columns. In both C_{18} methods, none of the columns was able to separate all the potential related substances from cefalexin under the prescribed LC conditions. The copolymer columns on the other hand showed good selectivity, independent of the origin or age of the columns. Four bulk samples of cefalexin were analysed by these three methods and the results were compared. Although the selectivity of polymer columns was better, for the samples examined no significant differences were observed for the assay results obtained with the three methods.

Introduction

Liquid chromatography (LC) on reversed-phase stationary phases based on silica, such as C_8 or C_{18}, often suffers from poor reproducibility of the selectivity. This was also observed in our laboratory, during analysis of cephalosporins (Wouters et al., 1984). Nevertheless, most of the pharmacopoeial methods precribe the use of these alkyl bonded stationary phases. For the assay of cefalexin the European Pharmacopoeia (Ph. Eur.) (European Pharmacopoeia, 1991) and the US Pharmacopeia (USP) XXII (US Pharmacopeia, 1989) prescribe different isocratic LC methods using C_{18} as stationary phase. The selectivity of these pharmacopoeial methods for cefalexin and a number of related substances was examined on different stationary phases in our laboratory. The chromatographic conditions were optimised following the prescribed suitability tests. Under the same conditions, four bulk samples were analysed. The results were compared to those obtained by an isocratic LC method using poly (styrene-divinylbenzene) (PSDVB) as stationary phase. This PSDVB method had been developed in our laboratory and was proved to give very satisfactory results for the reproducibility of the selectivity (Hendrix et al., 1993).

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Experimental

Reference substance and samples

The European Pharmacopoeia Chemical Reference Standard (93.4%) (Ph. Eur. CRS) was used as a standard. The bulk samples were chosen in order to have samples of variable purity.

Related substances

Related substances present as impurities in cefalexin can originate from the semi-synthesis and from degradation. The structures and origin of the potential impurities have been shown previously (Hendrix et al., 1993). 7-Aminodesacetoxycephalosporanic acid (**VII**) and D-phenylglycine (**VIII**) are the basic constituents of the cefalexin molecule. L-Cefalexin (**II**), Δ^2-cefalexin (**VI**) and phenylglycylcefalexin (**IX**) can arise from the semi-synthesis of cefalexin. The other related substances are decomposition products. 3-Hydroxymethylene-6-phenylpiperazine-2,5-dione (**III**) and 3-hydroxy-4-methyl-2(5H)-thiophenone (**IV**) are formed in acidic medium. **III** and 3-aminomethylene-6-phenylpiperazine-2,5-dione (**V**) are formed in neutral medium and the cefalexin Δ^4-cephalosporoates (**X**) are formed in alkaline medium. **X** was never isolated but was prepared in situ by dissolving cefalexin in 0.1 N NaOH (1 mg/ml) and storing the solution at room temperature for 10 min.

Solvents and reagents

Acetonitrile 99% (Janssen Chimica, Beerse, Belgium) and methanol (Roland, Brussels, Belgium) were distilled before use. Phosphoric acid 85% and potassium dihydrogen phosphate were from Merck (Darmstadt, Germany). Sodium 1-octanesulphonate 98% (NaOS), sodium 1-pentanesulphonate 98% (NaPS) and triethylamine 99% (Et_3N) were from Janssen Chimica. Water was distilled twice.

LC apparatus and operating conditions

Isocratic elution was used for the three methods. The equipment consisted of an L-6200 pump (Merck-Hitachi, Darmstadt, Germany), a 254 nm fixed wavelength UV monitor D (LDC/Milton Roy, Riviera Beach, FL, U.S.A.) and an integrator Model 3396 A (Hewlett-Packard, Avondale, PA, U.S.A.). The samples were injected by a Marathon autosampler (Spark Holland, Emmen, The Netherlands) with sample cooling (6°C) equipped with a fixed 20 μl loop and a cryomat Julabo C and F10 (Julabo Labortechnik, Seelbach, Germany). The columns (250 × 4.6 mm i.d.) were packed with: (A) RSIL C_{18} HL 10 μm (Bio-Rad, Eke, Belgium), (B) Partisil ODS-3 10 μm (Whatman, Clifton, NJ, U.S.A.), (C) Spherisorb ODS-1 10 μm (Phase Separations, Queensferry, U.K.), (D) Spherisorb ODS-2 10 μm, (E) Hypersil ODS 5 μm (Shandon, Runcorn, U.K.), (F) PLRP-S 100 Å 8 μm (Polymer Laboratories, Church Stretton, Shropshire, U.K.) or (G) PRP-1 7–9 μm (Hamilton, Reno, NV, U.S.A.). The columns were immersed in a water bath heated by a Julabo EM thermostat. The column temperature was 30°C for the alkyl bonded phases and 50°C for the PSDVB phases.

Mobile phases

The Ph. Eur. method prescibes as mobile phase a mixture having the following composition: methanol/acetonitrile/1.36% w/v solution of KH_2PO_4/water (2:5:10:83, v/v). The flow rate is 1.5 ml/min.

The mobile phase prescribed by the USP contains the following: water/acetonitrile/methanol/triethylamine/sodium 1-pentanesulphonate (850:100:50:15:1, v/v/v/v/w). The pH is adjusted to 3.0 with phosphoric acid. The flow rate is 1.5 ml/min.

The PSDVB method uses the following mixture: acetonitrile/0.02 M sodium 1-octanesulphonate/0.2 M phosphoric acid/water (15.5:10:5:up to 100, v/v). The flow rate is 1.0 ml/min.

Mobile phases were degassed by ultrasonication.

Sample preparation

Samples for quantitative analysis following Ph. Eur. or PSDVB methods were prepared by weighing 30 mg of cefalexin into a 20 ml volumetric flask. For the Ph. Eur. method water was used as solvent. The PSDVB method used a mobile phase containing 20% of the 0.02 M solution of

sodium 1-octanesulphonate as solvent. For the USP method 40 mg of cefalexin was weighed into a 100 ml volumetric flask and water was used as the solvent.

The chemical reference substance was dissolved the same way as the samples.

Results and Discussion

Examination of the selectivity of the LC methods

The selectivity of the pharmacopoeial methods was examined on five C_{18} columns (A–E) by the determination of the capacity factors of cefalexin and its related substances. For the Ph. Eur. method, for each column the composition of the mobile phase was adapted in order to achieve the required resolution of at least 4.0 between cefalexin and cefradine. Table 1 shows that all the columns examined complied with this requirement. The USP method also prescribes a resolution test as a criterion for the system suitability. Unfortunately, this test must be performed with cefalexin and the internal standard 1-hydroxybenzotriazole, which has a chemical structure totally different from that of cefalexin. In our opinion, this resolution criterion is not relevant for the separation of cefalexin and its related substances, which have structures very similar to that of the main compound. A resolution of at least 5 is required. The composition of the mobile phase and the corresponding resolution between cefalexin and 1-hydroxybenzotriazole for each column are listed in Table 2. The resolution test of the Ph. Eur. was also performed for this method

TABLE 1

Composition of the mobile phase and the corresponding resolution per column for the Ph. Eur. method

Mobile phase		Column				
		A	B	C	D	E
MeOH	ml	2	2	2	2	2
CH_3CN	ml	5	1.8	2.5	5	5
1.36% KH_2PO_4	ml	10	10	10	10	10
H_2O	ml	83	86.2	85.5	83	83
Resolution		4.5	4.1	4.6	4.9	10.1

TABLE 2

Composition of the mobile phase and the corresponding resolution (R_s USP) between cefalexin and 1-hydroxybenzotriazole per column for the USP method (R_s Ph. Eur., the resolution between cefalexin and cefradine)

Mobile phase		Column				
		A	B	C	D	E
MeOH	ml	40	25	28	40	40
CH_3CN	ml	80	50	56	80	80
NaPS	g	1	1	1	1	1
Et_3N	ml	15	15	15	15	15
H_2O	ml	880	925	916	880	880
R_s USP		5.4	5.7	5.3	7.4	17.0
R_s Ph. Eur.		3.4	3.4	3.8	4.5	7.9

and reported in Table 2. All the columns examined complied with the resolution test of the USP.

The results are shown in Figs 1 and 2. **X**, which is a complex mixture of diastereoisomers, is not shown in Figs 1 and 2. This polar mixture is eluted close to the front of the chromatogram. Using the chromatographic conditions described in Tables 1 and 2, neither method was able to separate completely cefalexin from all its related substances. Nevertheless, the chromatographic conditions comformed to the pharmacopoeial requirements. Significant differences in selectivity can be observed for the different columns.

The selectivity of the PSDVB method has already been examined and reported previously (Hendrix et al., 1993). This method is able to separate cefalexin from all its known related substances and performs equally well on different brands of PSDVB available on the market. The age and history (former use) of the columns were observed to have a limited influence on the selectivity. These characteristics are an important improvement compared to the pharmacopoeial methods.

Quantitative analysis of bulk samples by the three LC methods

Four bulk samples were analysed following the pharmacopoeial methods and following the PS-DVB method. The Ph. Eur. CRS of cefalexin was used as the standard.

The analyses following the Ph. Eur. and the USP were performed on the C_{18} columns A–E, using the same mobile phases as listed in Tables 1 and 2.

The results for the Ph. Eur. method are listed in Table 3. The relative standard deviation (RSD) calculated on the peak area of six subsequent injections of cefalexin was well below the prescribed limit of 1.0%. Each sample was analysed four times. For all the columns nearly the same results were obtained. The RSD did not exceed 1.0%.

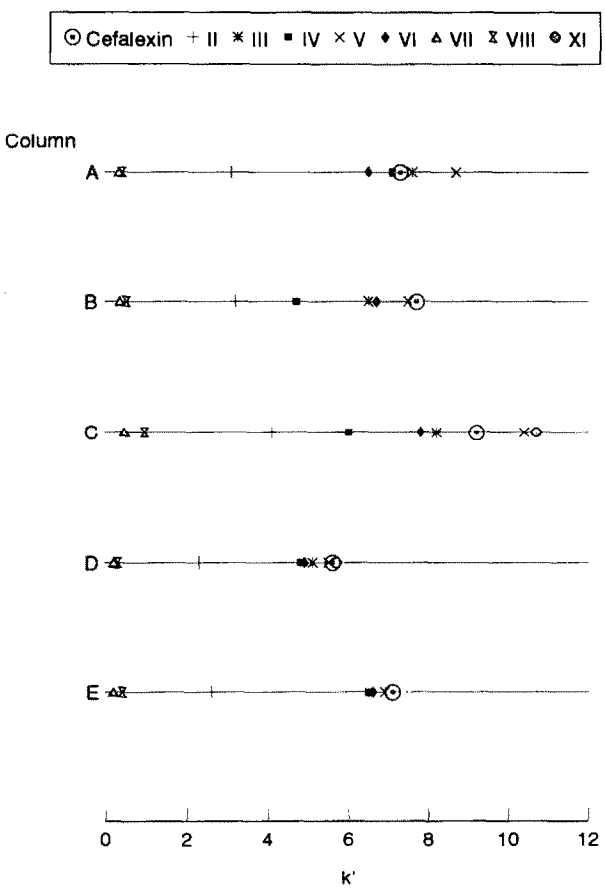

Fig. 1. Capacity factors of cefalexin and related substances for the LC method prescribed by the Ph. Eur. for the assay of cefalexin. Mobile phase, see Table 1; flow rate, 1.5 ml/min; column temperature, 30°C; UV detection at 254 nm; k' **IX** > 40, k' **XI** > 12 for columns A, B and E.

TABLE 3

Results of the assay following the Ph. Eur. method: values in % (w/w) (RSD values (%) are given in parentheses; number of analyses = 4)

Column	Sample			
	1	2	3	4
A	93.52	93.41	89.96	83.31
	(0.1)	(0.5)	(0.5)	(0.5)
B	92.98	92.72	90.06	82.59
	(0.1)	(0.6)	(0.3)	(0.3)
C	92.34	92.19	88.86	81.37
	(1.0)	(0.4)	(0.3)	(0.1)
D	92.90	92.97	89.82	82.51
	(0.2)	(0.4)	(0.1)	(0.2)
E	92.40	92.71	89.73	82.03
	(0.5)	(0.1)	(0.4)	(0.4)
Mean	92.88	92.80	89.69	82.36
$n = 20$	(0.6)	(0.6)	(0.6)	(0.9)

Table 4 shows the results for the USP method. The RSD for replicate injections ($n = 6$) of cefalexin did not exceed 0.8%, whereas the limit is 2.0%. The quantity of cefalexin was not calculated, as prescribed, by means of the internal standard, since an injector with a fixed loop volume was used during the analysis. The different columns gave comparable results. The RSD

TABLE 4

Results of the assay following the USP method: values in % (w/w) (RSD values (%) are given in parentheses; number of analyses = 4)

Column	Sample			
	1	2	3	4
A	92.85	92.74	89.84	81.72
	(0.3)	(0.4)	(0.3)	(0.3)
B	92.89	92.77	89.92	81.99
	(0.4)	(0.2)	(0.3)	(0.2)
C	91.69	91.55	88.61	80.84
	(0.1)	(0.1)	(0.2)	(0.1)
D	93.28	93.39	90.38	82.62
	(0.3)	(0.5)	(0.2)	(0.2)
E	92.29	92.22	90.11	81.68
	(0.2)	(0.2)	(1.3)	(0.1)
Mean	92.60	92.53	89.66	81.77
$n = 20$	(0.7)	(0.7)	(0.9)	(0.7)

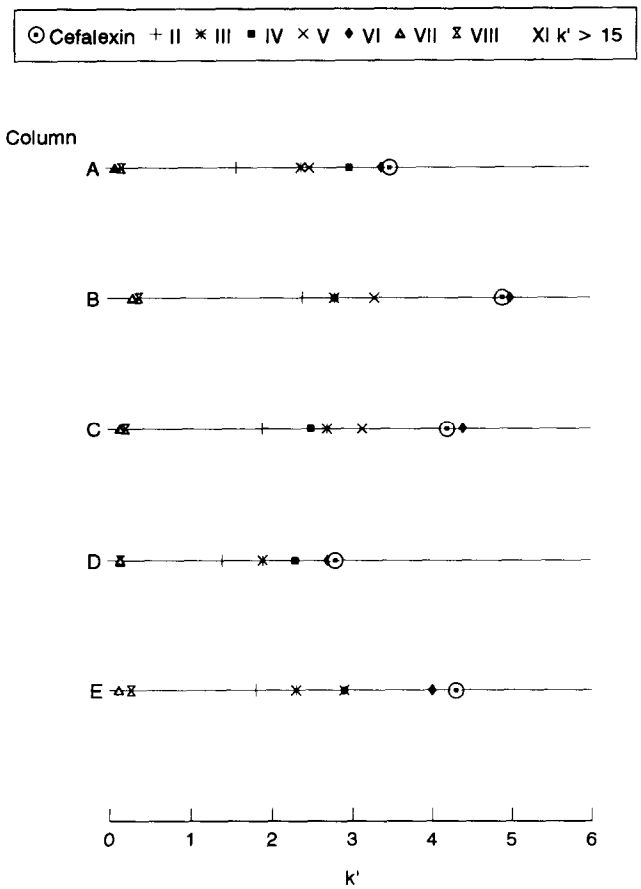

Fig. 2. Capacity factors of cefalexin and related substances for the LC method prescribed by the USP XXII for the assay of cefalexin. Mobile phase, see Table 2; flow rate, 1.5 ml/min; column temperature, 30°C; UV detection at 254 nm; k' **IX** > 40, k' **XI** > 15.

TABLE 5

Results of the assay following the PSDVB method: values in % (w/w) (RSD values (%) are given in parentheses; number of analyses = 4)

Column	Sample			
	1	2	3	4
F	92.56	92.71	89.41	81.84
	(0.1)	(0.4)	(0.5)	(0.3)
G	93.13	92.99	90.07	82.37
	(0.4)	(0.4)	(0.4)	(0.2)
Mean	92.85	92.85	89.74	82.11
$n = 8$	(0.4)	(0.4)	(0.6)	(0.4)

mostly was less than 1.0%. The results are approximately the same as for the Ph. Eur. method.

The analyses by the LC method developed on PSDVB were performed on columns F and G, which were the only brands of PSDVB stationary phases available on the Belgian market. Using the mobile phase described under Experimental, a resolution of 4.1 for column A and 4.4 for column B was obtained, which is better than the required resolution of 4.0 (Hendrix et al., 1993). The results are listed in Table 5. Comparable figures were obtained for both columns. The results are nearly the same as for the pharmacopoeial methods.

Conclusion

The results of the assay of four bulk samples by two pharmacopoeial LC methods using five different C_{18} columns and by an LC method using two different PSDVB columns were compared. The test of significance of differences of means (e.g., Spiegel et al., 1972) was performed using the grand means of the three methods. The resulting figures were less than the tabulated limits ($t_{0.995}$), therefore, the difference was not significant at this level. This conclusion, however is only applicable to the four samples examined, which apparently did not contain impurities which were coeluted with cefalexin in the pharmacopoeial methods. Nevertheless, the doubtful reproducibility of the selectivity of alkyl bonded stationary phases has once again been demonstrated here. Therefore, one should exercise caution in the use of LC methods using classical alkyl bonded stationary phases. These methods are less suitable as official methods. The PSDVB method, on the other hand, offers more reliable results because of its reproducible selectivity.

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