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A comparative study of LC methods for analysis of cefradine

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Abstract: A comparative study of two isocratic liquid chromatographic methods for the analysis of cefradine is described. The first method is prescribed by the European Pharmacopoeia for the assay of cefradine, using classical alkyl bonded phase (C_{18}) as the stationary phase. Poor reproducibility of the selectivity towards cefradine and its related substances was observed when this method was used and none of the C_{18} columns examined was able to separate cefradine completely from its potential related substances under the prescribed LC conditions. On the other hand, the second method, which uses poly(styrene-divinylbenzene) as the stationary phase, shows good selectivity even when using columns from different manufacturers and of different age. Four bulk samples of cefradine were analysed following both methods and the results were compared.

Keywords: *Cefradine; LC; reversed-phase; poly(styrene-divinylbenzene); method evaluation.*

Introduction

It has been observed that classical alkyl phases can suffer from poor reproducibility of the selectivity. This also occurred during liquid chromatography (LC) of cephalosporins using C_{18} columns [1]. However, this type of reversed-phase is widely used in pharmacopoeial methods. For the assay of cefradine the European Pharmacopoeia (Ph. Eur.) prescribes a LC method using C_{18} as stationary phase [2]. The United States Pharmacopeia (USP) XXII prescribes a similar method but only for the determination of the main related substance, cefalexin, while a hydroxylamine assay is used for cefradine [3]. The selectivity of the Ph. Eur. method for cefradine and its related substances was examined on six C_{18} columns. None of the columns gave complete separation of cefradine and its potential impurities. Only two of the six columns examined passed the prescribed system suitability test. Four columns were chosen for analysing four bulk samples following the Ph. Eur. method. The results were compared to those obtained by an isocratic LC method using poly(styrene-divinylbenzene) (PSDVB) as stationary phase. This PSDVB method has been developed in our laboratory and was proved to give satisfactory results for the reproducibility of the selectivity [4].

Experimental

Reference substances and samples

The European Pharmacopoeia Chemical Reference Standard (Ph. Eur. CRS; 93.5%) was used as the standard.

Bulk samples of different origin and age were chosen in order to have samples of variable purity.

Related substances

Cefalexin, which is formed from cefradine under influence of UV light, is the most important related substance and is always present in cefradine samples. Other related substances originate from the semi-synthesis and from degradation. The structures and origin of potential impurities of cefradine were shown previously [4]. 7-Aminodesacetoxycephalosporanic acid (**VII**) and D-cyclohexa-1,4-dienylglycine (**VIII**) are the basic constituents of the cefradine molecule. Δ^2-Cefradine (**VI**), cyclohexa-1,4-dienyl-glycylcefradine (**IX**) and the pivalamide of 7-ADCA (**XI**) can arise from the semi-synthesis of cefradine. The other related substances are decomposition products. 3-Hydroxymethylene-6-(cyclohexa-1,4-dienyl)-piperazine-2,5-dione (**III**) and 3-hydroxy-4-methyl-2(5*H*)-thiophenone (**VI**) are formed in acidic medium. **III** and 3-aminomethylene-6-(cyclo-

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hexa-1,4-dienyl)-piperazine-2,5-dione (**V**) are formed in neutral medium while cefradine Δ^4-cephalosporoates (**X**) are formed in alkaline medium. **X** was never isolated but was prepared *in situ* by dissolving cefradine in 0.1 N NaOH (1 mg ml^{-1}) 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. Sodium acetate (NaOAc) trihydrate was from Fluka Chemie (Buchs, Switzerland). Phosphoric acid 85%, acetic acid (HOAc) and sodium 1-octanesulphonate (NaOS) were from Janssen Chimica. Water was distilled twice.

LC apparatus and operating conditions

Isocratic elution was always used. The equipment consisted of a L-6200 pump (Merck–Hitachi, Darmstadt, Germany), a Merck–Hitachi L-4000 variable UV detector set at 254 nm and an integrator Model 3396 A (Hewlett–Packard, Avondale, PA). For the examination of peak homogeneity the UV detector was replaced by a photodiode array detector Model 990 (Waters Assoc., Milford, MA). 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 in the laboratory with (A) Hypersil ODS 5 µm (Shandon, Runcorn, UK), (B) Partisil ODS-3 10 µm (Whatman, Clifton, NJ), (C) Spherisorb ODS-1 10 µm (Phase Separations, Queensferry, Cheshire, UK), (D) RSIL C_{18} HL 10 µm (Bio-Rad, Eke, Belgium), (E) Nucleosil 100 C_{18} 5 µm (Macherey–Nagel, Düren, Germany), (F) Bio-Sil C_{18} LL 90 Å 5 µm (Bio-Rad), (G) PLRP-S 100 Å 8 µm (Polymer Laboratories, Church Stretton, Shropshire, UK) or (H) PRP-1 7–9 µm (Hamilton, Reno, NV). 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. For both methods the flow rate was 1 ml min^{-1}.

Mobile phase

The Ph. Eur. method prescribes a mixture of 2 M HOAc–3.62% m/v NaOAc–MeOH–H_2O (1:17:200:782, v/v/v/v).

The PSDVB method uses a mixture of acetonitrile–0.02 M sodium 1-octanesulphonate–0.2 M phosphoric acid–water (15:10:5:up to 100, v/v/v/v).

Mobile phases were degassed by ultrasonication before use.

Sample preparation

Samples for quantitative analysis following the Ph. Eur. method were prepared in mobile phase (25 mg/50 ml). For the PSDVB method the sample was dissolved (30 mg/20 ml) in mobile phase containing 20% of the 0.02 M sodium 1-octanesulphonate solution.

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

Results and Discussion

Examination of the selectivity of the LC methods

The selectivity of the Ph. Eur. method was examined on six C_{18} columns (A–F) by the determination of the capacity factors of cefradine and its related substances. For each column the composition of the mobile phase was adapted to obtain the required resolution of at least 7.0 between cefalexin and cefradine. Table 1 shows that only two of the columns examined (A and E) complied with this requirement. The resolution on the other columns was insufficient, even when cefradine was eluted very slowly (retention time >40 min). Nevertheless, the selectivity was investigated on each column. The mobile phase compositions were those marked with an asterisk in Table 1. The results are shown in Fig. 1. **X**, which is a complex mixture of diastereoisomers, is not shown in the figure. This polar mixture is eluted close to the dead volume of the chromatogram. As can be seen in Fig. 1, cefradine is not separated from all its related substances, even not on columns A and E which complied with the resolution test (R_s = 7.5 and 8.0, respectively). On columns A, C and F, only cefradine and Δ^2-cefradine (**VI**) were not separated. It should also be mentioned that there is insufficient relation between this resolution test and the selectivity of the LC method when applied to different columns. Differences in elution order can also be observed for the different columns, for instance the elution order of cefradine and **V** is

Table 1
Composition of the mobile phase and the corresponding resolution for each column following the Ph. Eur. method

Column	Mobile phase* Volumes MeOH	Volumes H_2O	Resolution
A	200	782	7.5†
B	120	862	3.6†
	60	922	4.3
C	130	852	4.3†
	80	902	5.6
D	160	822	3.4†
	100	882	5.2
E	200	782	8.0†
F	150	832	4.6†
	60	922	5.7

*The mobile phase further contains one volume of 2 M HOAc and 17 volumes of 3.62% m/v NaOAc.
†Mobile phase used for the selectivity test and for the quantitative analysis on columns A, C, E and F.

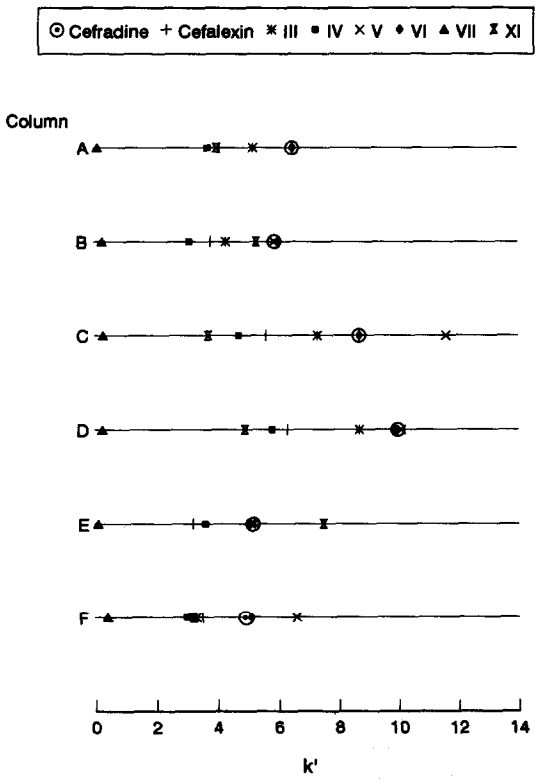

Figure 1
Capacity factors of cefradine and its related substances on different C_{18} columns obtained following the Ph. Eur. method. Mobile phases: see Table 1.

column dependent. The separation of cefradine from decomposition products formed in alkaline medium was examined by analysis of the *in situ* prepared solution of X. The homogeneity of the cefradine peak was examined by normalization at 260 nm of the UV spectra taken at the left slope, the maximum and the right slope. None of the columns gave a homogeneous cefradine peak. It can be concluded that the selectivity on C_{18} columns is poor.

The selectivity of the PSDVB method was already examined and reported previously [4]. This method demonstrates that cefradine can be separated from all its related substances. It is performing equally well on different brands of PSDVB available on the market. The age and the history (former use) of the columns was observed to have limited influence on the selectivity. These characteristics are an important improvement compared to the pharmacopoeial method.

Quantitative analysis of bulk samples by two LC methods

Four bulk samples were analysed following the pharmacopoeial method and the PSDVB method, respectively. The Ph. Eur. CRS for cefradine was used as the standard. This standard was also used for the determination of the related substance cefalexin because the results were only used for the evaluation of the two methods.

The analyses following the Ph. Eur. were performed on four C_{18} columns: A, C, E and F. The mobile phase compositions were those marked with an asterisk in Table 1. The results are listed in Table 2. The relative standard deviation (RSD) calculated on the peak area of six subsequent injections of cefradine CRS was well below the prescribed limit of 1.0% [2]. Each sample was analysed four times. With all the columns very similar results were obtained for the cefradine content. The RSD did not exceed 1.1%. The results for cefalexin were also quite reproducible. The RSD did not exceed 4.5%.

The analyses by the LC method on PSDVB were performed on columns G and H. Using the mobile phase described in the experimental section, a resolution of 4.3 for column G and 4.5 for column H was obtained between cefradine and cefalexin, which is better than the required resolution of 4.0 [4]. The results are listed in Table 3. Both columns gave nearly the same results for both cefradine and cefalexin. The RSD did not exceed 0.8 and 2.6%, respectively.

The results of the assay of four bulk samples by a pharmacopoeial method using four differ-

Table 2
Results of the assay following the Ph. Eur. method

	Sample							
	1		2		3		4	
Column	a	b	a	b	a	b	a	b
A	90.18	3.43	52.20	11.99	79.68	3.97	55.30	10.59
	(0.3)	(0.7)	(0.4)	(0.4)	(0.4)	(2.0)	(1.1)	(0.6)
C	90.31	3.69	53.38	12.98	80.30	4.36	55.99	11.58
	(0.6)	(0.5)	(0.4)	(0.3)	(0.1)	(0.9)	(0.6)	(0.5)
E	90.36	3.48	52.76	12.01	79.64	3.99	55.62	10.74
	(1.0)	(1.5)	(0.9)	(1.0)	(0.9)	(0.5)	(0.4)	(0.8)
F	90.16	3.68	52.83	13.02	80.32	4.30	55.58	11.56
	(0.8)	(0.7)	(0.3)	(0.5)	(0.3)	(0.6)	(0.3)	(0.1)*
Mean	90.25	3.56	52.79	12.50	80.03	4.15	55.62	11.09
	(0.6)	(3.4)	(1.0)	(4.2)	(0.6)	(4.5)	(0.7)	(4.3)
	$n = 16$	$n = 16$	$n = 16$	$n = 16$	$n = 16$	$n = 16$	$n = 16$	$n = 15$

Values in per cent (m/m); RSD values (%) are given in parentheses; number of analyses = 4. a, b represent cefradine and cefalexin, respectively.
*Number of analyses = 3.

Table 3
Results of the assay following the PSDVB method

	Sample							
	1		2		3		4	
Column	a	b	a	b	a	b	a	b
G	90.95	3.75	52.89	13.33	79.92	4.67	55.67	11.91
	(0.1)	(1.7)	(0.1)	(0.8)	(0.4)	(2.4)	(0.2)	(0.7)
	$n = 4$	$n = 4$	$n = 4$	$n = 4$	$n = 3$	$n = 4$	$n = 4$	$n = 4$
H	90.16	3.69	52.10	13.02	79.37	4.51	54.82	11.60
	(0.8)	(2.6)	(0.4)	(0.2)	(0.3)	(0.5)	(0.2)	(0.8)
	$n = 4$	$n = 4$	$n = 4$	$n = 4$	$n = 4$	$n = 3$	$n = 4$	$n = 4$
Mean	90.56	3.72	52.49	13.17	79.60	4.61	55.25	11.75
	(0.7)	(2.2)	(0.8)	(1.4)	(0.5)	(2.6)	(0.8)	(1.6)
	$n = 8$	$n = 8$	$n = 8$	$n = 8$	$n = 7$	$n = 7$	$n = 8$	$n = 8$

Values in per cent (m/m); RSD values (%) are given in parentheses; n = number of analyses. a, b represent cefradine and cefalexin, respectively.

ent C_{18} columns and by a method using two different PSDVB columns were compared. The test of significance of difference of means [5] was performed using the grand means of both methods. The resulting figures were less than the tabulated limits ($t_{0.001}$) for both cefradine and cefalexin, so there is no significant difference at 99.9% level between these two methods.

Conclusions

The results of the assay seem to lead to the conclusion that both methods are equivalent. However, this can be confirmed only for the samples examined, which apparently did not contain impurities which were coeluted with cefradine in the pharmacopoeial method.

In general it can be concluded that the poor reproducibility of the selectivity of the classical alkyl bonded phase columns was observed here again. Therefore, methods which use alkyl bonded phases are less suitable as official methods. The PSVB method on the other hand, offers more reliable results because of its reproducible selectivity.

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