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Multidimensional evaluation of impurity profiles for generic cephalexin and cefaclor antibiotics

B.A. Olsen*, S.W. Baertschi and R.M. Riggin

Lilly Research Laboratories, Eli Lilly and Company, P.O. Box 685, Lafayette, IN 47902 (USA)

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ABSTRACT

A multidimensional approach is described for characterizing impurities in samples of generic cefaclor and cephalexin antibiotics. High-performance liquid chromatography (HPLC) with gradient elution followed by photodiode array or mass spectrometric detection provides valuable information concerning the nature of impurities observed. Results are presented which demonstrate the utility of these techniques for identifying impurities and distinguishing among process-related impurities, degradation products and formulation excipients. Preparative HPLC isolation and spectroscopic identification of some impurities is also described.

INTRODUCTION

High-performance liquid chromatography (HPLC) is extremely useful for the determination of related substances in cephalosporin antibiotics [1,2]. Related substances are defined as structurally related impurities arising from the manufacturing process or by degradation [3]. Determination of these impurities in bulk drug substances and products is important for quality control and evaluation of samples from different sources. For evaluation of drug quality, it is desirable to determine not only the total amount of related substances, but the nature or identity of these impurities. This is especially important if impurity profiles are qualitatively different between samples. Such differences may be caused by changes in the manufacturing process or a switch in supplier of the bulk drug. Different manufacturing processes may give rise to different process-related impurities, and the treatment of the product during manufacturing and storage by different suppliers may produce a variable profile of degradation products. The "fingerprinting" of drug products using multiple techniques has recently been proposed as a way to monitor and detect such changes [4]. Another aspect of profiling impurities in formulated products such as oral suspensions is the need to distinguish between excipients and impurities.

While HPLC can provide much information regarding impurity profiles, to better meet the needs outlined above, information beyond a single-wavelength absorbance *vs.* time chromatogram is required. Capacity factor data together with ultraviolet–visible spectra from photodiode array (PDA) detection have been used for confirmation of peak identity in toxicological drug screening [5–7], analysis of forensic samples [8–10], drug metabolism studies [11–14] and evaluation of impurities in bulk drugs and formulations [15–18]. Liquid chromatography–mass spectrometry (LC–MS) has also been utilized for positive peak identification and quantification in drug disposition studies [11,19–22] and in confirmation of drug impurity or degradation product identities [23,24]. In this paper, a multi-

* Corresponding author.

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dimensional approach utilizing HPLC with PDA and/or MS detection as tools for drug impurity characterization is described. Preparative HPLC isolation and structural characterization by nuclear magnetic resonance (NMR) spectroscopy is also employed when necessary. The approach is illustrated by examination of impurity profiles for cephalexin and cefaclor bulk drug substance and formulated product from different sources.

EXPERIMENTAL

Reagents

HPLC-grade acetonitrile and methanol were obtained from EM Science (Gibbstown, NJ, USA). Mobile phases were prepared using monobasic sodium phosphate monohydrate, also from EM Science. Water for mobile phase and sample solutions was purified with a Milli-Q system from Millipore (Milford, MA, USA). Samples of bulk and formulated cefaclor were obtained from Eli Lilly & Co. and generic manufacturers.

Apparatus

The chromatographic system consisted of a Model 600E pump (Waters, Bedford, MA, USA) and a Model 728 autoinjector (Alcott, Norcross, GA, USA) with a 20-μl fixed-loop injection valve (Valco, Houston, TX, USA). Single-wavelength detection using a Model 787 UV detector (Applied Biosystems, Ramsey, NJ, USA) set at 220 nm was used for most samples. Single-wavelength chromatograms were recorded using an in-house data acquisition system. When UV spectra of sample components were desired, a Waters Model 990 PDA detector was used in place of the single-wavelength detector. All UV spectra given in the figures were acquired using PDA detection of components after HPLC separation. The amplitude of some spectra has been scaled numerically for ease of comparison in the figures.

The HPLC separation was performed on a 250 mm $\times$ 4.6 mm, 5 μm particle size, YMC-ODS column (YMC, Morris Plains, NJ, USA) using 50 m*M* sodium phosphate, pH 4.0, with a two-stage acetonitrile gradient from 2.25 to 45% [1]. The sample concentration for bulk cephalexin and cefaclor was 5.0 mg/ml. Formulated samples were prepared based on the dosage strength to give concentrations of cephalexin or cefaclor of approximately 5 mg/ml. This concentration provided an injection of 100 μg of antibiotic onto the analytical HPLC column.

The LC–MS experiments were performed with a Beckman System Gold liquid chromatograph (Beckman Instruments, Palo Alto, CA, USA) and a Sciex Model API III mass spectrometer (Sciex, Thornhill, Canada). A heated nebulizer atmospheric chemical ionization source was used for the work described here. The ionspray mode has also been used for similar samples. The mobile phase for LC–MS contained 0.1% acetic acid instead of phosphate buffer. The mobile phase was held at 15% acetonitrile for 15 min followed by a linear ramp to 25% acetonitrile over 10 min. A Beckman Model 168 diode array UV–Vis detector was used between the column and mass spectrometer interface to detect any changes in retention order from the phosphate eluent system. Flow was diverted away from the MS source during elution of the cefaclor peak to reduce cefaclor background in subsequent scans. The source was held at 450°C with nitrogen used as the nebulizer gas at a pressure of 80 p.s.i. (1 p.s.i. = 6894.76 Pa). For these experiments, 200 μl of a 5 mg/ml cefaclor sample solution were injected (1 mg applied to the column).

Preparative isolation

The following conditions were used to isolate impurities from source C cefaclor (see Results and Discussion). The mobile phase for preparative HPLC isolation consisted of a gradient from 25 to 50% solvent B over 18 min after an initial isocratic period (5 min at 25% solvent B). Solvent A consisted of an aqueous solution of 0.1% acetic acid, while solvent B consisted of a mixture of acetonitrile–water–acetic acid (60:40:0.1, v/v/v). The flow-rate was 20 ml/min. A YMC-ODS (250 $\times$ 20 mm, 5 μm particle size) column was used.

The sample was dissolved in 0.025 *M* HCl at a concentration of 10 mg/ml. A total of 40 ml of this solution was injected onto the column for each preparative run (38 preparative runs were made altogether). Three fractions were col-

lected. These fractions were cooled to 0°C immediately upon collection until all 38 preparative runs were completed. The fractions were then frozen and lyophilized to remove solvents. A small aliquot of the remaining solids from each fraction was analyzed by gradient HPLC using the cefaclor-related substances method [1] prior to spectroscopic characterization.

NMR analysis

The NMR spectra were collected on a Bruker 500 MHz NMR system (Bruker Instruments, Billerica, MA, USA) in [$^2H_6$]dimethyl sulfoxide (with a trace of trifluoroacetic acid added to slow down proton exchange and sharpen the resonances), and referenced to internal tetramethylsilane. Carbon–proton correlation assignments were detected using two-dimensional heteronuclear experiments designed to detect correlations due to coupling through one [25] or more than one [26] chemical bond.

RESULTS AND DISCUSSION

Characterization process

To survey the impurities present in cephalexin or cefaclor, a gradient HPLC profile at 220 nm where nearly all related substances will show some absorbance is obtained. This can provide quantitative results for individual and total impurities [1]. Identities of several components may be known from retention time match with known impurities and past experience of which impurities are likely to be present. Matching UV spectra from a PDA detector for a known impurity and a peak of interest with the same retention time provides a strong, if not absolute indication of peak identity.

Most process-related impurities in cephalexin and cefaclor contain an intact 3-cephem nucleus while degradation products do not [1,27]. Therefore, if the spectrum of an unknown component has the characteristic 3-cephem absorbance maximum at about 260–265 nm [28] the component is likely to be a process-related impurity. Other spectral features indicate a degradation product or possibly an excipient in a formulation sample. A sample may be characterized further by obtaining structural information from LC–MS or

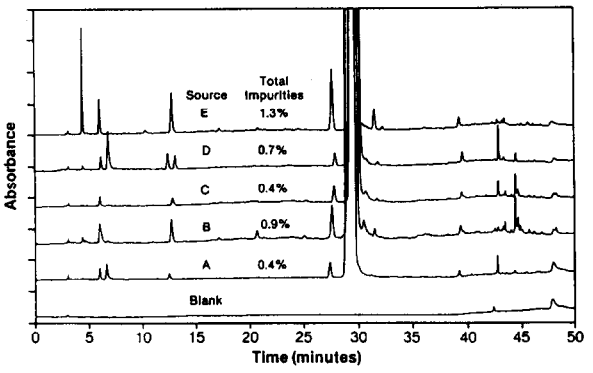


Fig. 1. Impurity profiles of bulk cephalexin from multiple sources. Wavelength 220 nm.

from spectroscopic measurements after preparative HPLC isolation. This can provide absolute identification of previously unknown impurities. This characterization process adjusted for the spectral properties of the compounds being examined could be applied to the analysis of several drugs.

Bulk drug substance analysis

Impurity profiles for bulk cephalexin from several suppliers are shown in Fig. 1. The total level of impurities is comparable among these samples, but there are differences in the individual components present. The chromatogram for the source D sample showed a retention time match with two known impurities, Δ^2-cephalexin (isomer nomenclature follows the cephem ring numbering convention [29]) and N-phenylglycyl cephalexin (Fig. 2). As shown in Fig. 3, the UV

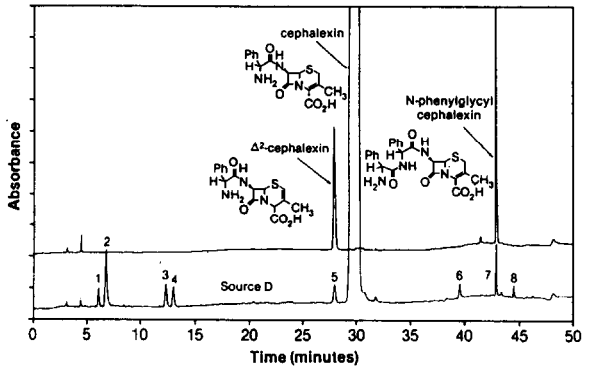


Fig. 2. Retention time comparison of source D cephalexin impurities with Δ^2-cephalexin and N-phenylglycyl cephalexin. Wavelength 220 nm. Approximately 0.4 μg each of Δ^2-cephalexin and N-phenylglycyl cephalexin injected.

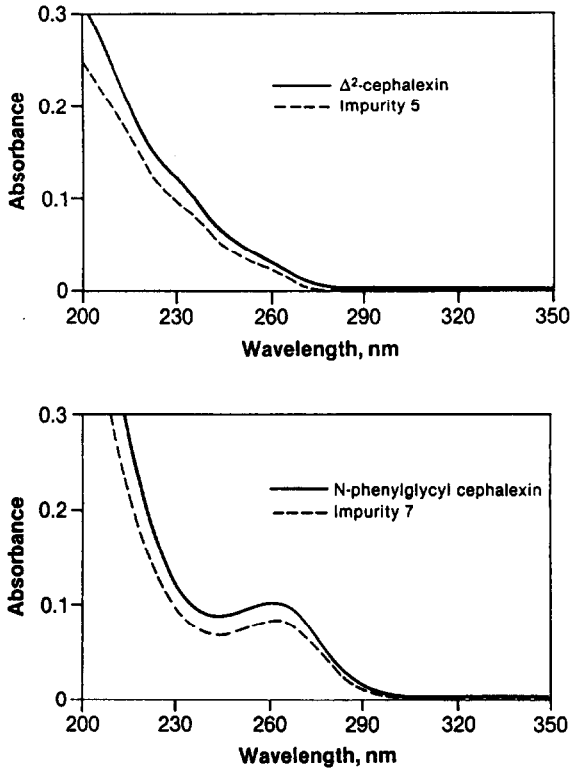


Fig. 3. UV spectra comparison of source D cephalexin impurities with Δ^2-cephalexin and N-phenylglycyl cephalexin.

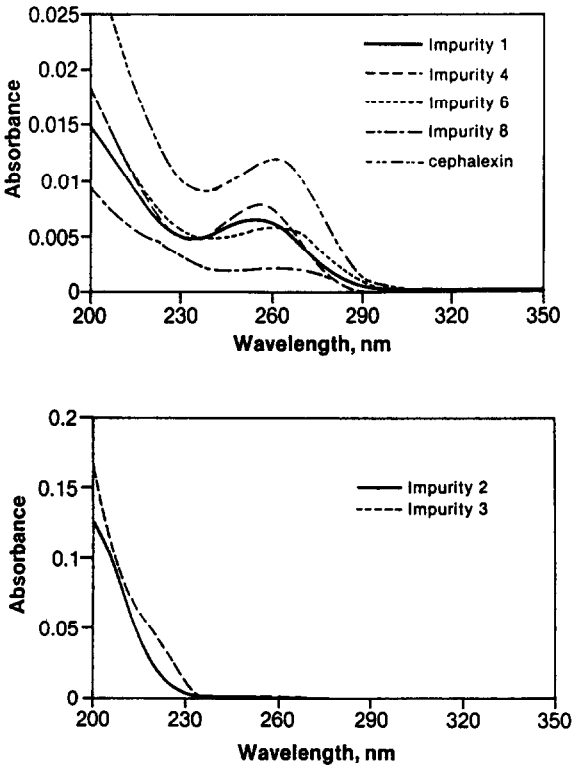


Fig. 4. UV spectra of unknown impurities in source D cephalexin.

spectra for the sample impurities also matched the known impurity spectra, thereby confirming their identities. The Δ^2-isomer is a primary degradation product of cephalexin and has a spectrum much different from that of cephalexin. In contrast, the N-phenylglycyl cephalexin impurity is process-related, resulting from over-acylation during the final synthetic step. Its spectrum is nearly identical to that of cephalexin. Spectra for unknown impurities (Fig. 4) indicated that four are likely to be process-related because of the 3-cephem absorbance band (peaks 1, 4, 6, 8) and two are probably degradation products (peaks 2, 3).

Impurity profiles for bulk cefaclor shown in Fig. 5 revealed three late-eluting impurities in one sample that were not present in the others. These impurities accounted for a large portion of the higher level of total related substances compared to the other samples. The expanded chromatogram in Fig. 6 shows that the retention time of peak 1 matched that of N-phenylglycyl cefaclor (PG-cefaclor) and peak 2 was very close in retention to 2-hydroxy-3-phenylpyrazine (2,3-HPP), a known degradation product. A match of the UV spectra was obtained for PG-cefaclor, but the peak 2 spectrum was much different from

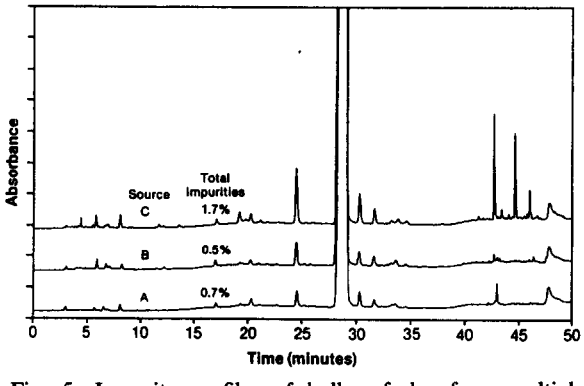


Fig. 5. Impurity profiles of bulk cefaclor from multiple sources. Wavelength 220 nm.

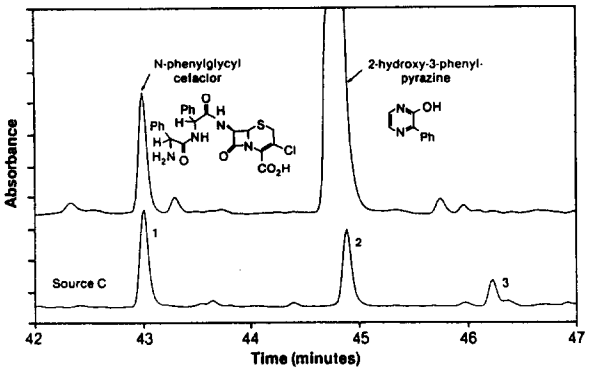


Fig. 6. Expanded chromatogram comparing retention of source C cefaclor impurities with N-phenylglycyl cefaclor and 2-hydroxy-3-phenylpyrazine. Wavelength 220 nm. A 0.6-μg amount of N-phenylglycyl cefaclor and 4 μg 2-hydroxy-3-phenylpyrazine injected.

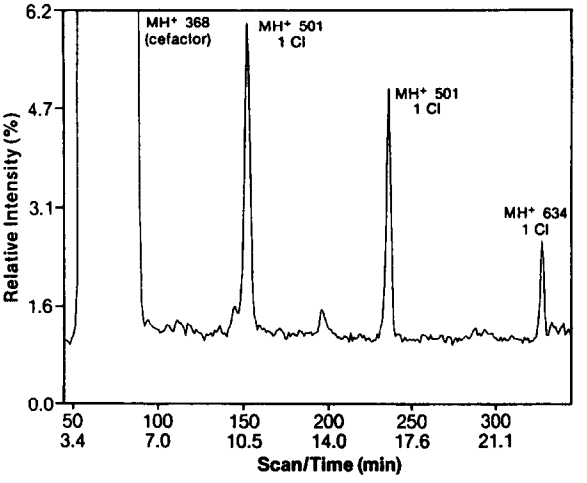


Fig. 8. Total ion current LC–MS chromatogram for late-eluting impurities in source C cefaclor.

that of 2,3-HPP (Fig. 7). Spectra of peaks 2 and 3 were nearly identical to that of PG-cefaclor, suggesting that they were also process-related impurities.

The source C cefaclor sample was analyzed by LC–MS–MS using atmospheric pressure chemical ionization followed by collison-induced dissociation of the molecular ion for fragmentation information. The protonated molecular ions from each peak of the total ion current chromatogram are indicated in Fig. 8 with an example of MS–MS fragmentation data for the first impurity peak shown in Fig. 9. The identification of PG-cefaclor by retention time and UV spectral match was further confirmed by the MH^+ ion at m/z 501 and appropriate fragment ions. Impurity peaks 2 and 3 gave MH^+ ions corresponding to cefaclor plus one and two phenylglycine groups, respectively. Without careful control of

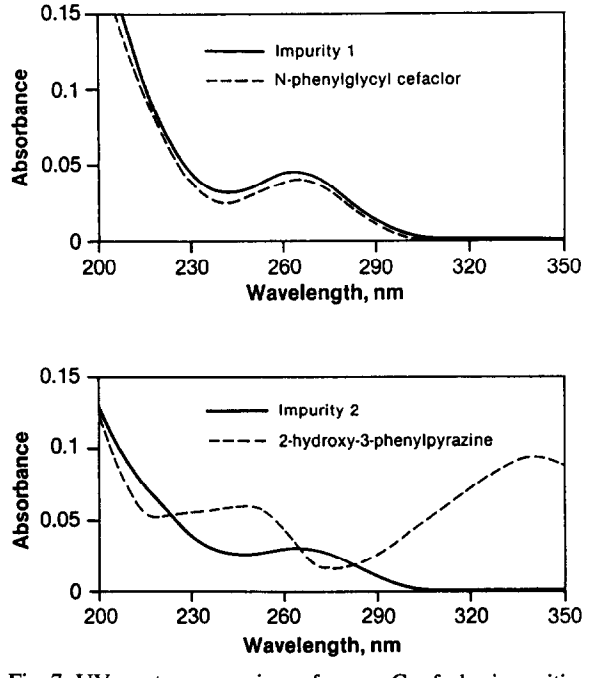


Fig. 7. UV spectra comparison of source C cefaclor impurities with N-phenylglycyl cefaclor and 2-hydroxy-3-phenylpyrazine.

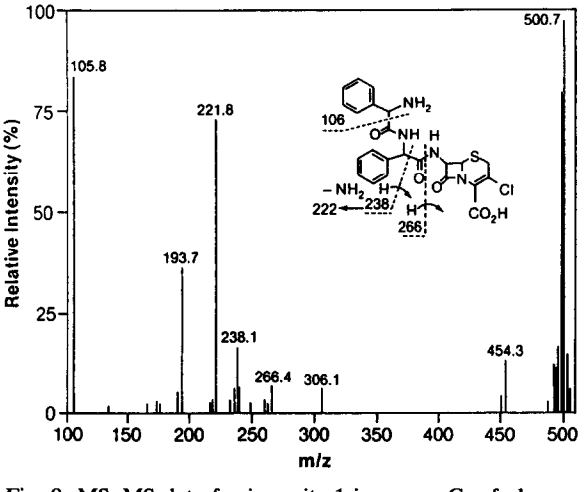


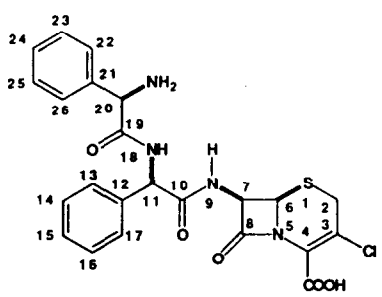
Fig. 9. MS–MS data for impurity 1 in source C cefaclor.

the reaction conditions used to prepare cefaclor, acylation at sites other than the desired 7-amino group on the 3-chloro nucleus may take place. Adding another phenylglycine to the phenylglycine side chain of a cefaclor molecule forms PG-cefaclor. Adding yet another phenylglycine to the side chain gives a compound with a molecular mass corresponding to that observed for impurity 3. During the acylation reaction it may have also been possible to form an acid chloride at the carboxylic acid on the 3-chloro nucleus. This species could then form an amide with phenylglycine in addition to being acylated at the 7-amino position. This compound would have the same molecular mass as PG-cefaclor, but with the extra phenylglycine at a different position. The mass spectral data for impurities 2 and 3 supported the structures given in Fig. 10.

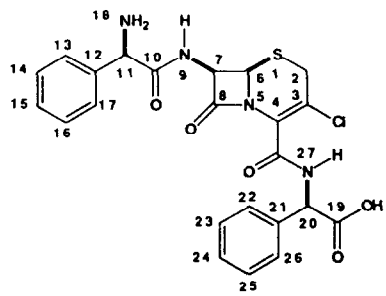
Additional confirmation of the molecular structure of impurity peaks 1–3 was obtained by preparative HPLC isolation and off-line characterization by NMR. Conditions for isolation and NMR analysis are contained in the Experimental section. Chemical shift assignments of the proton and carbon NMR spectra for the three isolated impurites are given in Table I. These data confirm the structures as those given in Fig. 10.

Formulation analysis

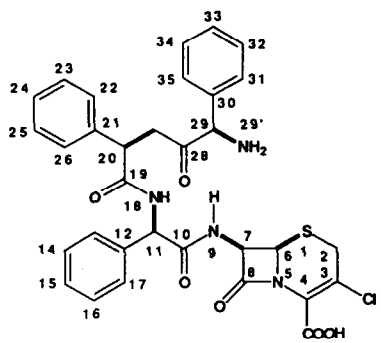
The presence of excipients in formulations such as oral suspensions adds the complication of distinguishing peaks due to excipients from those due to impurities. If the excipients in a formulation are known, their retention and UV response under the gradient HPLC conditions can be compared to suspect peaks in the sample. Even



Impurity 1: N-phenylglycyl cefaclor



Impurity 2: C-phenylglycyl cefaclor



Impurity 3: N,N'-diphenylglycyl cefaclor

Fig. 10. Structures of impurities 1–3 in source C cefaclor.

TABLE I

^1H AND ^{13}C NMR ASSIGNMENTS OF CEFACLOR AND IMPURITIES FROM SOURCE C (IN [$^2H_6$]DIMETHYL SULFOXIDE)

^{13}C first line; ^1H second line; values in ppm *vs.* tetramethylsilane. n.a. = Not assigned; n.m. = not measured; – = not applicable.

Site[a]	Cefaclor	1	2	3
2	30.07	n.m.	29.72	29.88
	3.87, 3.58	3.85, 3.58	3.78, 3.49	3.86, 3.59
2-COOH	161.61	n.m.	159.57	161.57
	–	–	–	–
3	121.03	n.m.	118.03	120.91
	–	–	–	–
4	125.56	n.m.	127.48	125.30
	–	–	–	–
6	57.29	n.m.	57.32	57.33
	5.15	5.07	5.08	5.08
7	58.90	n.m.	58.74	58.58
	5.82	5.69	5.70	5.69
8	163.17	n.m.	162.24	163.69
	–	–	–	–
9	–	n.m.	–	–
	9.64	9.40	9.64	9.33
10	168.45	n.m.	168.57	170.08
	–	–	–	–
11	55.69	n.m.	55.56	55.83
	5.06	5.67	5.04	5.55
12	133.57	n.m.	133.66	137.59
	–	–	–	–
13, 17	128.06	n.m.	n.a.	127.07
	7.54	n.a.	n.a.	7.29
14, 16	128.91	n.m.	n.a.	n.a.
	7.44	n.a.	n.a.	n.a.
15	129.54	n.m.	n.a.	n.a.
	7.44	n.a.	n.a.	n.a.
18	–	–	–	–
	8.78	9.32	8.72	9.06
19	–	n.m.	171.14	168.74
	–	–	–	–
20	–	n.m.	56.45	55.83
	–	5.12	5.48	5.82
21	–	n.m.	137.05	138.07
	–	–	–	–
22, 26	–	n.m.	n.a.	127.31
	–	n.a.	n.a.	7.29
23, 25	–	n.m.	n.a.	n.a.
	–	n.a.	n.a.	n.a.
24	–	n.m.	n.a.	n.a.
	–	n.a.	n.a.	n.a.
27	–	–	–	–
	–	8.63	–	9.20
28	–	–	–	166.79
	–	–	–	–
29	–	–	–	55.16
	–	–	–	5.11

(Continued on p. 172)

TABLE I *(continued)*

Site[a]	Cefaclor	1	2	3
30	–	–	–	133.99
	–	–	–	–
31, 35	–	–	–	127.86
	–	–	–	7.43
32, 34	–	–	–	n.a.
	–	–	–	n.a.
35	–	–	–	n.a.
	–	–	–	n.a.
36	–	–	–	–
	–	–	–	8.60

[a] See Fig. 10 for numbering.

without knowing what excipients were used, their distinctive absorption spectra can often indicate their presence.

Chromatograms for two cefaclor oral suspensions are shown in Fig. 11. An unknown component designated as peak 1 was observed in the source A sample. FD&C Red 40 was listed as a coloring agent on the package insert for this sample and the retention time for this compound matched that of the unknown peak. The UV spectrum of FD&C Red 40 also clearly matched that of the unknown peak (Fig. 12). The large late-eluting component (peak 2) in sample B was identified by retention time and UV spectral match (Fig. 12) as benzoate, a common preservative, even though this ingredient was not listed on the package or package insert. Identification of these peaks as excipients justified not including them as impurities and allowed an

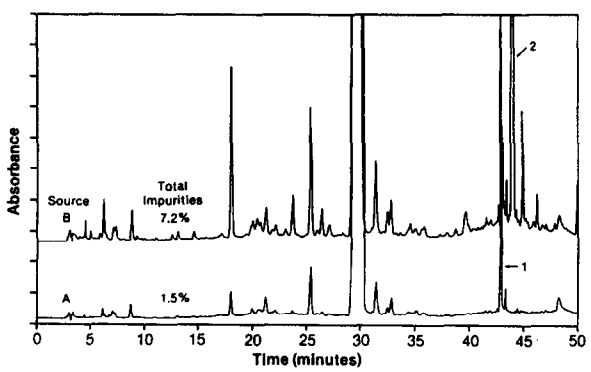


Fig. 11. Impurity profiles of cefaclor oral suspension formulations from two sources. Wavelength 220 nm. UV spectra for unknown peaks 1 and 2 are given in Fig. 12.

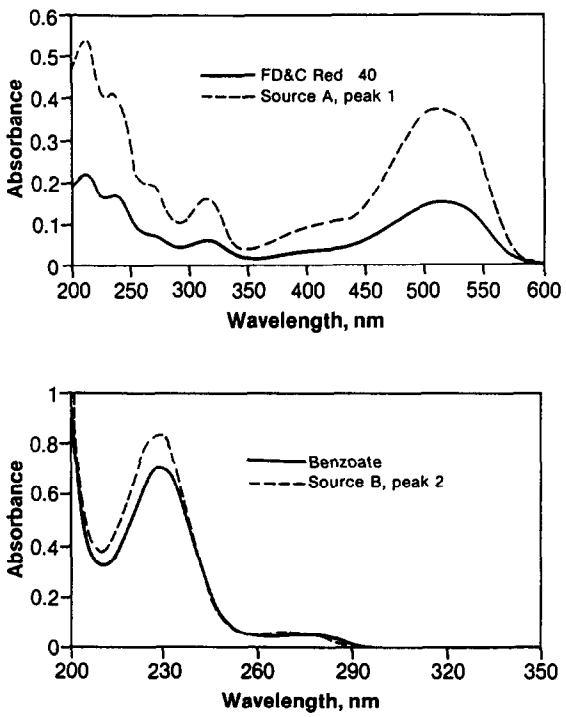


Fig. 12. Comparison of excipient and sample component UV spectra.

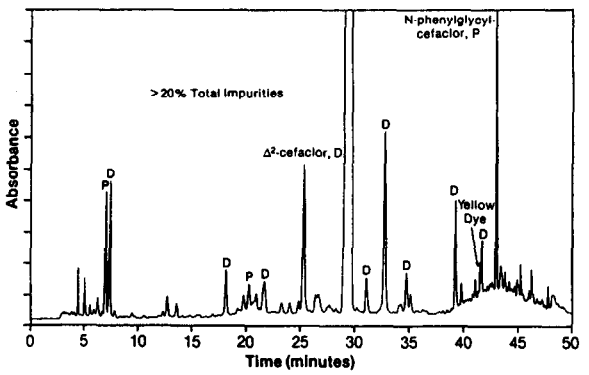


Fig. 13. Impurity profile for cefaclor granule formulation. Wavelength 220 nm. P = Process-related impurity; D = degradation product.

accurate assessment of total impurities in the samples.

Another example of formulation analysis is the impurity profile of a cefaclor granule formulation shown in Fig. 13. Some peaks were identified, while others were characterized as process-related impurities or degradation products as indicated. This example shows the utility of the approach for a sample containing a large number of impurities.

CONCLUSIONS

For HPLC analysis of impurities in cephalexin and cefaclor, PDA detection can be used to confirm impurity identity, provide an indication of whether an impurity is process-related or a degradation product, and distinguish excipients from impurities. LC–MS can also be used to obtain structural information useful in identification of unknowns. While the experimental conditions would change for different products, the general approach used here for cephalexin and cefaclor can be applied to the analysis of many drugs.

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