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Short Communication

High-performance liquid chromatographic determination of cephalosporin antibiotics using 0.3 mm I.D. columns

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ABSTRACT

Four cephalosporins, cefazolin, ceftizoxime, cefaloridine and cefaclor, were determined using a novel microbore high-performance liquid chromatographic system designed to be entirely compatible with direct liquid interfacing (DLI) for mass spectrometric analysis. The chromatographic support was a 5-μm C_{18} column of 0.3 mm I.D., compared with the usual microbore column diameters of 1–2 mm. The mobile phase contained no buffers or salts which may have caused column blocking or mobile phase crystallization, and the use of a concentration column allowed the injection of large volumes of analyte (up to 500 μl). The assay was reproducible, the relative standard deviations being less than 20% within-day and between-day for all the drugs. The detection limit for cefaloridine and cefazolin was 1 ng and for cefaclor and ceftizoxime 5 ng.

INTRODUCTION

The occurrence of death due to shock following the use of cephalosporin antibiotics has been encountered in medico-legal practice, hence the development of a high-performance liquid chromatographic–mass spectrometric (HPLC–MS) system for the determination of these drugs in body fluids was necessary. For this purpose, the preferred interface was direct liquid introduction (DLI), which placed various physical and chemical constraints on the properties of the LC system required.

When interfacing mass spectrometers with HPLC systems directly, it is usual to use a splitter valve to reduce the pressure at which the mobile phase enters the spectrometer, resulting in the loss of part of the analyte. An alternative approach is to use super-microbore column chromatography, which allows the analytes to be chromatographed at an extremely low flow-rate, and hence low pressure. In this way, the whole of the mobile phase can be interfaced with the mass spectrometer. This, however, prohibits the use of buffer salts, particularly phosphates, as the width of the tubing linking the various parts of the equipment is so small that blockage caused by

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mobile phase crystallization is a major problem. Further, flow-rates must be kept below a maximum of 10 μl/min to ensure that the pressure does not exceed the acceptable maximum for interfacing with the mass spectrometer.

Conventional HPLC systems for the determination of cephalosporins have been reported. Most of these are limited in the range of drugs which they can determine [1–5], detection by fluorimetry [6] or insufficient detection limits [7–9]. Most of these methods also include the use of phosphate or acetate buffer salts.

One microbore HPLC method for cephalosporins has been reported [10], but this uses phosphate buffer in the mobile phase and no information was given on the UV detection wavelength or detection limits. The advantages of using microbore columns were, however, pointed out; these include a substantial of reduction in the column packing material and solvent consumption (as much as 95%), and a 16-fold increase in sensitivity over conventional columns. In this paper, we report a 10-fold increase in sensitivity over our conventional HPLC procedure [11].

EXPERIMENTAL

Chemicals and reagents

Cefazolin and ceftizoxime were supplied by Fujisawa Pharmaceutical (Japan) and cefaclor and cefaloridine by Shionogi Pharmaceutical (Japan). All solvents were of HPLC grade.

Drug standard solutions

Standard solutions of the pure drugs were made up in distilled water at concentrations of 0.01, 0.05, 0.1, 0.25 and 0.5 μg/ml.

Chromatographic equipment and conditions

The super-microbore HPLC system consisted of two pumps, A and B. Pump A (Milton Roy, U.S.A.) was used to deliver mobile phase A [deionized water–methanol–acetic acid (60:40:0.5)] at a rate of 4 μl/min over the analytical column. Pump B (Jasco, Tokyo, Japan) was used to pass mobile phase B (0.01 *M* ammonium acetate solution adjusted to pH 5 with acetic acid) as the wash solution for the loop and concentration column at a rate of 0.1 ml/min.

The analytical column (15 cm × 0.3 mm I.D.) contained 5-μm ODS C_{18} packing and the concentration column (3 cm × 0.3 mm I.D.) contained 5-μm ODS C_{18} packing. Both columns were supplied by Nomura Chemical (Seto, Japan).

The injection system (Rheodyne) incorporated a 500 -μl fixed loop with a tubing dead volume of 50 μl.

The variable-wavelength UV detector (Jasco) monitored the eluent at 262 nm, and a computer integrator (Shimadzu) was used to collect the chromatographic data.

The tubing used to connect the sections of the system was of 0.05 mm I.D.

Analytical procedure

Accurate volumes of analyte (100 μl) were injected into the loop followed by deionized water (at least 50 μl) to fill the dead volume. The system was such that, in the first instance, mobile phase B was allowed to pass through the loop onto the concentration column, where the analyte was trapped. After 3 min, a switching valve

allowed mobile phase A to pass over the concentration column, causing back-flushing of the analyte from the precolumn onto the main column for determination. The capacity of the loop was 500 μl (maximum injection volume).

Optimization

Mobile phase B was tested at pH 3, 5, 7 and 9. The slightly acidic pH 5 was found to give the best recovery from the concentration column.

The waiting time between delivery of the analyte onto the concentration column and its removal by mobile phase A onto the analytical column was optimized at 3 min (flow-rate 0.1 ml/min). A wait of 5 min resulted in the analyte being completely washed from the column; a wait of 2 min did not allow the system sufficient time to deposit the analyte on the column.

RESULTS AND DISCUSSION

Main analytical column

A typical chromatogram of the four cephalosporins is shown in Fig. 1. The peak shape was excellent for all drugs. The detector response (peak height) was linear for all drugs over the range 0.05–0.5 μg/ml and all graphs passed through the origin (Fig. 2). The retention times were ceftizoxime 5.75, cefaclor 6.71, cefaloridine 7.03 and cefazolin 8.50 min.

The relative standard deviations, both within-day ($n=3$) and between-day ($n=3$), were less than 20% in all instances, and generally the reproducibility increased with increasing concentration, suggesting that the cephalosporins are more unstable at low than at higher concentrations.

The detection limits were 1 ng for cefazolin and cefaloridine on-column (2 ng/ml) and 5 ng for cefaclor and ceftizoxime (10 ng/ml). This is about a 10-fold increase in sensitivity compared with the previously reported conventional HPLC method [11].

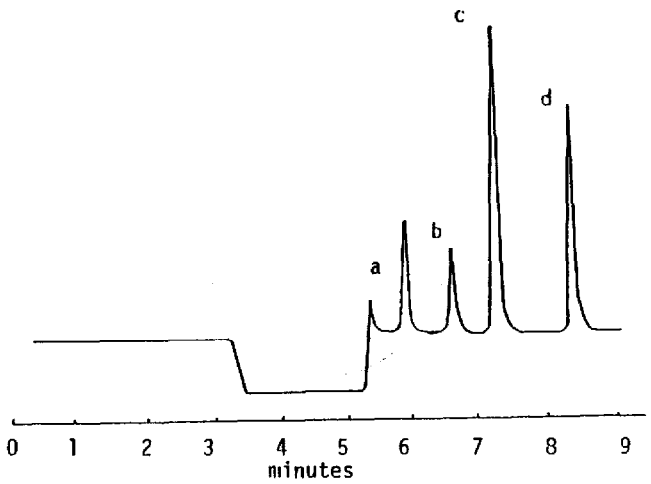

Fig. 1. Cephalosporin standards (0.1 μg/ml; 100 μl). Mobile phase, deionized water–methanol–acetic acid (60:40:0.5); flow-rate, 4 μl/min; detection wavelength, 262 nm; C_{18} column, 15 cm × 0.3 mm I.D. Samples: (a) ceftizoxime; (b) cefaclor; (c) cefaloridine; (d) cefazolin.

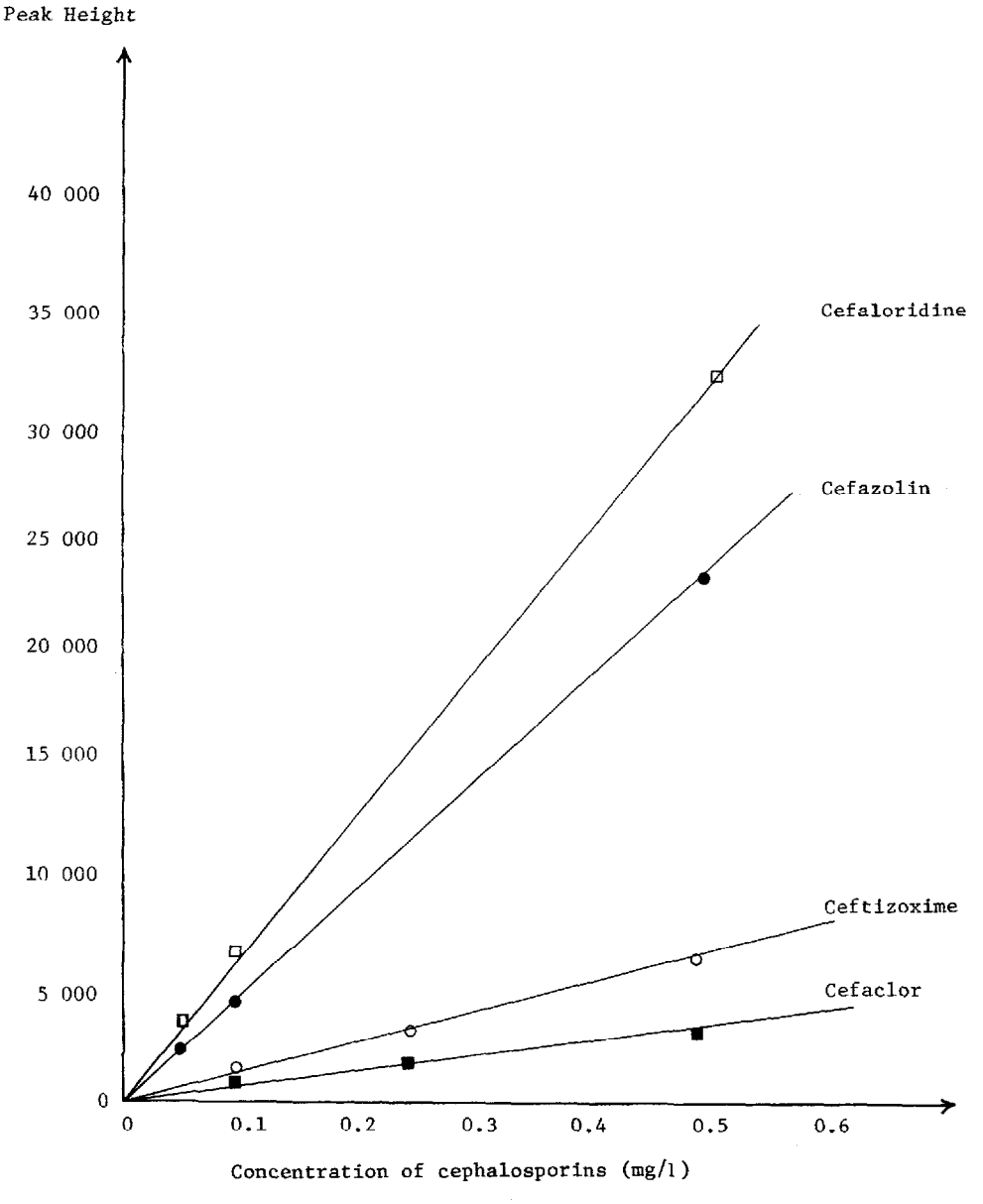

Fig. 2. Linearity of detector response to various drug concentrations. Mean values are shown.

Concentration column

The interactions between the acidic cephalosporin drugs (Fig. 3) and the non-polar C_{18} packing of the concentration column are mostly hydrophobic. Hence the acidic aqueous mobile phase (B) aids the retention of the analytes on the concentration column.

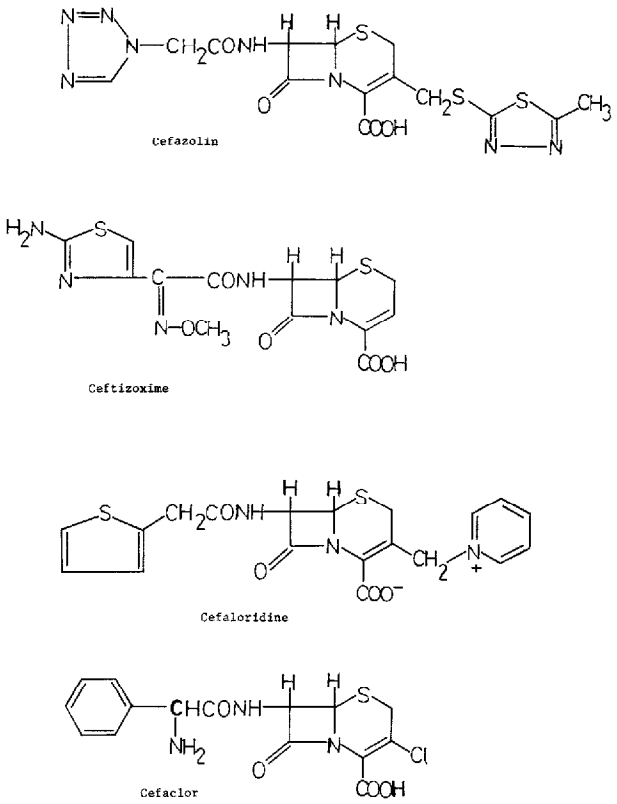

Fig. 3. Structures of the cephalosporins investigated.

The less polar mobile phase (A) which contains 40% methanol removes the drugs from the concentration column and carries them to the main analytical column for determination.

The use of a concentration column circumvents the most common problem associated with the use of microbore columns, *i.e.*, low injection volumes. Using this highly efficient retention/elution system, up to 500 μl of isolate matrix can be analysed.

The purpose of this work was to develop a reproducible HPLC–MS procedure for the determination of cephalosporins. The super-microbore method described is, in itself, suitable for the determination of such antibiotics, as each of the drugs could easily be used as an internal standard for the others. However, the interfacing with a mass spectrometer gives an additional identification procedure and, as we wished to use fast atom bombardment as the fragmentation process, the incorporation of a fluid matrix in the mobile phase was necessary. The addition of glycerol (1%) to the mobile phase shortened the retention times and reduced the peak heights of the cephalosporins.

CONCLUSION

The reproducibility and efficiency of this super-microbore liquid chromatographic system make it ideal for interfacing with a mass spectrometer by direct liquid introduction. Buffer salts and high flow-rates are not necessary. The use of the concentration column allows the injection of up to 500 μl of analyte, so the detection limits, even when glycerol is incorporated in the mobile phase, are easily sufficient to detect therapeutic levels of cephalosporins. The observed decrease in retention time when glycerol was added to the mobile phase caused the cephalosporins to elute closer together, but when mass spectrometry is applied this is not a problem, as separation and resolution using mass chromatograms is possible.

The possibilities of mass spectrometer interfacing are now under investigation.

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