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Short Communication

Column-switching high-pressure liquid chromatographic method for the determination of a new carbapenem antibiotic, L-739,428, in rat and monkey plasma

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Abstract

A column-switching HPLC method for determination of a new carbapenem antibiotic, L-739,428 (I), was developed that allowed direct injection of rat and monkey plasma. Following dilution with a pH 6.5 buffer, samples were injected without further cleanup into an extraction column dry-packed with 10-μm particle size Maxsil C_{18} reversed-phase adsorbent. Endogenous plasma components were washed to waste for 6 min with a weak mobile phase of 0.025 *M* sodium phosphate, 0.005 *M* hexanesulfonate (pH 6.5). Compound I, retained on the extraction column, was then backflushed with a mobile phase which consisted of a mixture of the preceding buffer-ion pair solution and acetonitrile (96:4, v/v) into a Partisphere C_{18} analytical column and detected by ultraviolet absorption at 299 nm. Chromatographic retention time was 11.5 min. Stability of I in plasma was maximized by use of a refrigerated autosampler which maintained plasma at 5°C until analyzed. The limit of quantification was 0.1 μg/ml using the equivalent of 75 μl plasma.

1. Introduction

L-739,428 (BO-2727, I, Fig. 1) is a new carbapenem with Gram-positive and Gram-negative antibiotic activity *in vitro* that may have use in the treatment of clinical bacterial infections. To minimize the possible introduction of analytical artifacts through excessive sample manipulation, a direct injection column-switching HPLC method was developed for determination of I in rat and monkey plasma. Another carbapenem antibiotic, imipenem, exhibits limited stability in biological fluids and special precautions are necessary to minimize artifactual degradation during analytical procedures [1]. Direct injection HPLC methods are useful for analytes with finite stability because conditions (pH, temperature, darkness) frequently can be established under which degradation is blocked or sufficiently slowed to allow unattended analysis

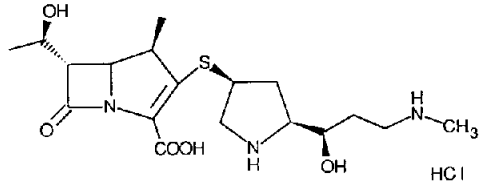

Fig. 1. Structure of L-739,428 (I).

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[2–5]. An additional benefit of direct injection methods over traditional off-line cleanup methods (*i.e.* liquid–liquid or liquid–solid) is elimination of labor intensive sample preparation.

2. Experimental

2.1. Chemicals and reagents

Compound I was synthesized by Banyu Laboratories (Tokyo, Japan). Chromatography-grade acetonitrile (Fisher, Fair Lawn, NJ, USA) and water (Millipore, Milford, MA, USA) were used in preparation of mobile phases. Molecular biology grade monobasic and dibasic sodium phosphate were obtained from J.T. Baker (Phillipsburg, NJ, USA). Empty stainless steel HPLC columns and 1-hexanesulfonate sodium were purchased from Alltech (Deerfield, IL, USA). 2-(N-Morpholino)ethanesulfonate (MES) was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of the highest grade commercially available.

2.2. Equipment and chromatographic conditions

A Spectra-Physics Model 8800 pump and 8880 autosampler (equipped with cooling unit), and Waters M6000A pump were used with a two-position six-port switching valve (Autochrom, Milford, MA, USA) to provide automated on-line extraction and analysis (Fig. 2). Detection was by UV absorption at 299 nm using a Model 785A detector from Applied Biosystems (Foster City, CA, USA). The Turbochrome computer program (Version 3.2, PE Nelson, Cupertino, CA, USA) was used for chromatographic data acquisition and reduction.

Extraction columns with dimensions of 50 × 4.6 mm I.D. were dry-packed in the author's laboratory with a reversed-phase adsorbent (Maxsil C_{18}, 10-μm particle size; Phenomenex, Torrance, CA, USA). Since use of different adsorbents in extraction and analytical columns frequently provides increased selectivity, also examined as potential adsorbents in the extraction column were cyano, C_8, and anion-exchange

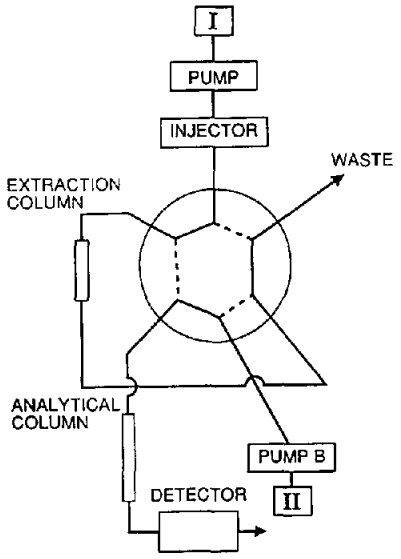

Fig. 2. Schematic diagram of column-switching system. Flow path of mobile phases was changed from wash (solid line) to backflush (dotted line) using a two-position six-port switching valve. Plasma was injected into the extraction column and endogenous components washed to waste with mobile phase A (see text). Compound I was backflushed from the extraction to the analytical column with mobile phase B.

packing materials. However there was insufficient analyte retention with these adsorbents under the conditions examined. The analytical column was a reversed-phase Partisphere C_{18} 5-μm 125 × 4.6 mm I.D. (Whatman, Clifton, NJ, USA) preceded by a Brownlee RP-18 7-μm 15 × 3.2 mm I.D. guard column (ABI, Foster City, CA, USA).

Mobile phase (A) for the extraction column consisted of 0.025 *M* sodium phosphate with 0.005 *M* 1-hexanesulfonate sodium (adjusted to pH 6.5) at a flow-rate of 2.5 ml/min. The analytical mobile phase (B) was a mixture of the preceding buffer-ion pair solution and acetonitrile (96:4, v/v) pumped at a flow-rate of 2.2 ml/min with typical backpressure of 14 MPa. Mobile phases were filtered daily and continuously degassed with helium.

Following injection of plasma, the extraction column was washed with mobile phase A for 6 min to remove plasma proteins and other endogenous interferences. From 6 to 8 min, the switch-

ing valve changed the flow path of mobile phase to backflush I from the extraction column into the analytical column with mobile phase B. Afterwards, the mobile phase flow path was returned to initial conditions (run time 14 min). Retention time of I was 11.0 to 11.8 min under these conditions. The 6-min wash time was chosen because initial studies showed that using the extraction column with mobile phase A alone, a large peak corresponding to plasma proteins and other endogenous compounds eluted by 5 min. Under the same conditions, I eluted as a broad peak with a retentiontime of 48 min. In preliminary studies, accumulation of particulates in biological fluids led to increased backpressure (>30 MPa) and shortened useful life of the extraction column. A semi-preparative filter frit (1.9-cm diameter, 2-μm porosity; Upchurch, Oak Harbor, WA, USA) placed downstream from the autosampler permitted continuous injection of at least 60 samples without increase in backpressure.

Working solutions of 2, 5, 10, 20, 50, 100, 250, 500 1000, and 2000 μg/ml were prepared in 1 *M* MES buffer (pH 6.5). Standards were prepared by mixing 50 μl of working solutions with 950 μl control plasma for a calibration curve range from 0.1 to 100 μg/ml. Similarly, quality control samples containing I in final concentration of 7.5, 15, and 30 μg/ml were prepared from working solutions of 150, 300, and 600 μg/ml (bulk powder was weighed separately from that used for calibration solutions). Calibration curves were constructed by linear regression of peak area *vs.* concentration using a weighting factor of $1/Y$, chosen empirically because it resulted in the smallest relative errors.

2.3. Stability in plasma

To determine proper conditions for handling of plasma samples from pharmacokinetic studies, stability of I (25 μg/ml) was examined at 5 and 37°C in freshly drawn rat and monkey plasma. The effect of adding one volume of 1 *M* MES (pH 6.5) buffer to plasma was evaluated because previous work indicated maximal aqueous solution stability at pH 6–7 (not shown). To determine suitable storage conditions, stability in plasma frozen at −70°C was examined over one month.

2.4. Accuracy, precision, and recovery

Accuracy and precision of the method for analysis of rat and monkey plasma were assessed through quintuplicate analysis of calibration standards and quality control samples containing I in known concentration. Evaluation of inter-day performance was through replicate analysis of quality control samples containing I in final concentration of 7.5, 15, and 30 μg/ml over three days. Mean relative error was expressed as the percentage difference between the mean assayed concentrations and nominal values. Recovery was assessed by comparing peak areas after injection of plasma with those resulting after injection of drug in buffer solution directly into the analytical column. Calibration standards were prepared fresh daily and maintained on ice throughout.

2.5. Sample preparation

Plasma (150 μl) was thawed on wet ice and pH adjusted by addition of 1 volume ice-cold 1 *M* MES (pH 6.5) buffer. Following centrifugation at 10 000 *g* for 10 min at 5°C to remove gross particulates, 150 μl of the supernatant was directly injected onto the chromatographic system. The refrigerated autosampler maintained samples at 5°C until analysis. Suitability of the method for *in vivo* studies was established through analysis of plasma from rats and monkeys drawn subsequent to i.v. administration of 60 mg/kg doses of I.

3. Results and discussion

3.1. Chromatography

The column-switching system in which I was adsorbed to a C_{18} extraction column and then backflushed to a C_{18} analytical column provided adequate cleanup of plasma as shown by the

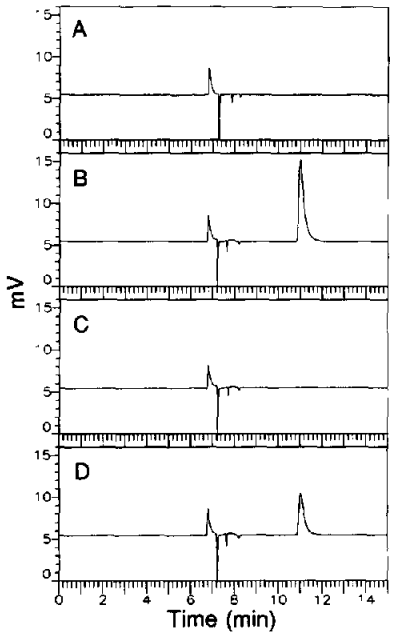

Fig. 3. Representative chromatograms of rat and monkey plasma. Shown are (A) monkey plasma prior to, and (B) 4 h after administration of a 60 mg/kg i.v. dose of I, rat plasma (C) prior to, and (D) 1 h after i.v. administration of the same dose. Detector output: one volt equaled one absorbance unit.

absence of interfering peaks in predose plasma (Fig. 3). The C_{18} extraction column was inexpensively self-packed and allowed analysis of *ca.* 200 samples (routinely replaced after 150 samples) before increased peak width and decreased analyte retention necessitated replacement. Deterioration of the extraction column presumably resulted from the irreversible adsorption of endogenous plasma components. After more than 500 samples, chromatography on the analytical column remained satisfactory, although backpressure increased by 1.4 MPa.

3.2. Stability

Stability of I was limited in fresh rat and monkey plasma at 37°C such that after 60 min >20% was degraded in rat and monkey plasma, while cooling to 5°C improved stability such that ≥97% was maintained over the same time period. In rat plasma, cooling alone was insufficient to permit overnight unattended analysis as indicated by a 15% decrease in peak area over 15 h. Stability was further enhanced by combining cooling with addition of 1 volume 1 *M* MES (pH 6.5) buffer to plasma. The combination of cooling and pH adjustment maintained >96% of the initial amount for at least 15 h. Frozen rat and monkey plasma containing I showed no evidence of degradation over storage for 30 days at −70°C.

3.3. Accuracy, precision, and recovery

Recoveries of I from rat and monkey plasma at 2.5 and 25 μg/ml ranged from 95.0 to 99.1%. An assessment of assay performance carried out using the conditions of greatest stability (cooling to 5°C and addition of one volume pH 6.5 buffer) indicated that the method was sufficiently accurate and precise. During quintuplicate analysis of calibration standards over the 0.25 to 100 μg/ml range in rat and monkey plasma, coefficients of variation were <6.6% and mean relative errors ranged from −6.9 to 6.4%. Mean relative error was 10% in rat and monkey plasma at a concentration of 0.1 μg/ml, which was taken as the limit of quantification. Inter-day accuracy and precision were satisfactory as assessed by replicate analysis ($n = 15$) of quality control samples over three days (Table 1).

3.4. Application in pharmacokinetic studies

The procedure was applied in single i.v. dose pharmacokinetic studies in rats and rhesus monkeys. At defined intervals for 2 h in rats and 6 h in monkeys after i.v. administration of 60 mg/kg, blood samples were drawn into precooled tubes, immediately centrifuged, and the plasma was rapidly frozen on dry ice. No interference from endogenous components was observed in any of the predose plasma samples. At these dose levels, terminal half-lives of I were 14 min in rats and 63 min in monkeys (Fig. 4). Plasma concentrations remained above the 0.1 μg/ml quantification limit for the entire sampling period. Since plasma concentrations should be followed for at least four half-lives for optimal characterization of the area under the concen-

Table 1
Accuracy and precision of the method during analysis of rat and monkey plasma quality control samples

Nominal concentration (μg/ml)	Rat plasma		Monkey plasma	
	Relative error (%)	Coefficient of variation (%)	Relative error (%)	Coefficient of variation (%)
7.5	−2.5	3.8	1.1	3.7
15	−3.7	2.4	0.9	1.5
30	−1.6	2.4	0.7	3.2

Assays were conducted in quintuplicate over three days (total $n = 15$).

tration–time curve, sensitivity of the method was more than sufficient for the intended use.

4. Conclusion

A direct injection column-switching HPLC method was developed for quantification of I in rat and monkey plasma. Adjusting sample pH and use of a refrigerated autosampler maximized stability of I. Diluted plasma was directly injected into a C_{18} extraction column and endogenous plasma components washed to waste. I was then backflushed to a C_{18} analytical column and detected by ultraviolet absorption at 299 nm. The method provided sufficient sensitivity (lower limit of quantification of 0.1 μg/ml), accuracy, and precision for pharmacokinetic studies of I in laboratory animals.

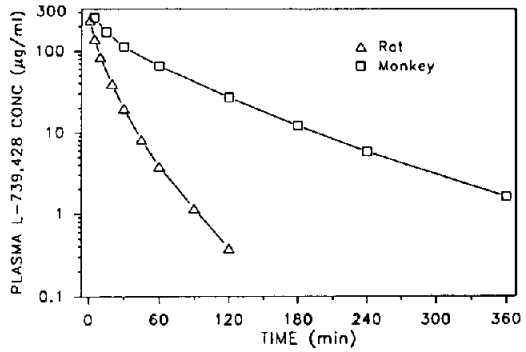

Fig. 4. Plasma concentration–time profile in rats and monkeys following single dose i.v. administration of 60 mg/kg.

5. References

1. D.A. Gravellese, D.G. Munson, Pauliukonis and W.F. Bayne, *J. Chromatogr.*, 310 (1984) 71.
2. F. Erni, H.P. Keller, C. Morin and M. Schmitt, *J. Chromatogr.*, 204 (1981) 65.
3. T. Ohkubo, M. Kudo and K. Sugawara, *J. Chromatogr.*, 573 (1992) 289.
4. U. Timm, G. Hopfgartner and R. Erdin, *J. Chromatogr.*, 456 (1988) 21.
5. R. Wyss and F. Bucheli, *J. Chromatogr.*, 593 (1992) 55.