Journal of Chromatography, 375 (1986) 444—450
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 2933

Note

Determination of N-formimidoylthienamycin concentration in sera from pediatric patients by high-performance liquid chromatography

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(First received July 30th, 1985, revised manuscript received October 28th, 1985)

Of the several carbapenem antibiotics described in the scientific literature, the best characterized to date is thienamycin [1] Produced during the growth of *Streptomyces catellya*, it is bactericidal for a wide variety of microorganisms [2, 3] As investigations progressed, the compound was modified to increase its chemical stability resulting in N-formimidoylthienamycin (NFT), a broad-spectrum β-lactamase stable antibiotic [4—6] Early animal and human pharmacokinetic studies revealed NFT to be metabolized by the renal brush border enzyme dehydropeptidase I, resulting in poor drug recovery from urine and nephrotoxicity in rabbits [6]. When coadministered with an inhibitor of this enzyme, cilastatin sodium, urinary recovery increased sufficiently to allay suspicion of ineffectiveness in treating urinary tract infections [7, 8] Imipenem is an equal weight combination of NFT and cilastatin sodium that is currently undergoing clinical trials The chemical structure of NFT is depicted in Fig 1 [9, 10]

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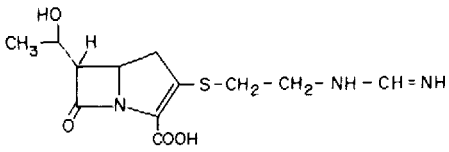

Fig 1 Structure of N-formimidoylthienamycin

First-dose pharmacokinetics of imipenem in children and steady-state pharmacokinetics in adults and patients with cystic fibrosis have been described [11—14] A major problem encountered during pediatric drug studies involves adequate sample collection Two high-performance liquid chromatographic (HPLC) assays for imipenem have been described in the literature [15, 16] Both use expensive micropartition filtration systems for deproteinization of serum or plasma and specify testing of large sample volumes (50—75 μl) We report an HPLC assay that includes an economical extraction method and requires small sample volumes (8 μl) for testing.

EXPERIMENTAL

Materials

HPLC-grade acetonitrile and reagent-grade boric acid were obtained from Fisher Scientific (King of Prussia, PA, U S A.). 5-Methoxyindole-3-acetic acid (MIAA) was purchased from Sigma (St Louis, MO, U S A.) Pooled drug-free human serum (Q-Pak) was obtained from Hyland Diagnostics (Deerfield, IL, U.S.A.), NFT was provided by Merck, Sharp and Dohme Research Labs (Rahway, NJ, U.S A) Centrifree micropartition system filtration units (1 ml) were obtained from Amicon (Danvers, MA, U S.A.)

Apparatus

The HPLC system consisted of a Series 10 single-piston, rapid-fill pump and an LC-85B variable-wavelength UV spectrophotometric detector which was equipped with a 1 8-μl flow cell Chromatograms and calculations were carried out with an LCI-100 laboratory computing integrator All of the above were maufactured by Perkin-Elmer (Norwalk, CT, U S A.). Sample injections were made into a 7125 syringe loading sample injector equipped with a 20-μl sample loop. Both were manufactured by Rheodyne (Cotati, CA, U S.A)

Chromatographic conditions

Separations were made using an HS-5, 125 mm × 4 mm I D analytical column packed with 5-μm C_{18} reversed-phase material (Perkin-Elmer) A 3-cm guard column was filled with 5-μm C_{18} pellicular material and attached directly to the analytical column

Pump flow-rate was 3 5 ml/min Injection volume was 8 μl manually delivered to the injection valve via a partially filled 25-μl Hamilton syringe The detector was set at 300 nm with a response time of 200 ms The run time was 5 5 min The LCI-100 integrator was programmed for the percent method using a chart speed of 5 mm/min, attenuation of 4, peak width of 7, area sensitivity of 16, baseline sensitivity of 23 and the skim sensitivity was set at

0. The scale of the integrator was calibrated from −0.2 mV to 70 mV for a detector output of 1 mV. All assays were carried out at room temperature

Prepared solutions

Sterile 2 *M* 2-(N-morpholino)ethanesulfonic acid (MES), pH 6 5 serum-stabilizing solution in concentrated form was provided by Merck, Sharp and Dohme and was stored at 1—2°C MES stock solution was diluted 1 1 with deionized water to prepare a 1 *M* working solution Each serum sample was stabilized by the addition of an equal volume of the MES working solution

Mobile phase for the assay was 0 2 *M* boric acid buffer adjusted to pH 7 2 with 10 *M* sodium hydroxide. The solution was degassed by sparging with helium

Standard preparation

NFT powder was dissolved in MES to obtain a stock solution of 1000 μg/ml Serial dilutions were prepared at concentrations ranging from 2 to 1000 μg/ml The solutions were vortexed, aliquoted and frozen at −70°C until used for standard preparation

Serum standards were prepared by adding 0 1 ml of thawed, vortexed NFT stock solution to 0.9 ml of drug-free serum to obtain final serum concentrations ranging from 0 2 to 100 μg/ml After vortexing, an equal volume of MES was added to each serum standard. The stabilized serum specimens were again vortexed, divided into 0 5-ml aliquots and frozen at −70°C

Prior to HPLC assay, deproteinization of 0 5-ml aliquots of the stabilized serum standards was accomplished by the addition of 0.5 ml of acetonitrile followed by 20 min of agitation. An internal standard, MIAA, was added to the acetonitrile to obtain a final serum concentration of 544 μg/ml

Following deproteinization, the standards were centrifuged for 20 min at 1000 *g*, the supernatants were removed and frozen at −70°C Immediately prior to HPLC analysis the standards were thawed, centrifuged and placed in an ice bath Specimens separated into aqueous and acetonitrile phases, 8 μl of the aqueous (bottom) phase were injected into the HPLC system for analysis

During a second phase of the study, serum standards were deproteinized by a micropartition method. Aliquots (1 ml) of MES-stabilized NFT serum standards containing MIAA as an internal standard were placed into the micropartition device and centrifuged in a fixed-angle rotor for 30 min at 1000 *g* at room temperature.

RESULTS

Linearity and reproducibility

Peak-area and peak-height data were collected for NFT and MIAA Serum determinations were made using the log of the NFT/MIAA peak-area ratios compared to standard curves The linearity of log peak-height ratio and log peak-area ratio response versus log NFT/MIAA concentration ratio was determined by linear regression The means of triplicate standard assays were linear between 0 8 and 100 μg/ml with correlation coefficients of 0 996 and 0 995 for peak-area and peak-height ratios, respectively The average coefficient of

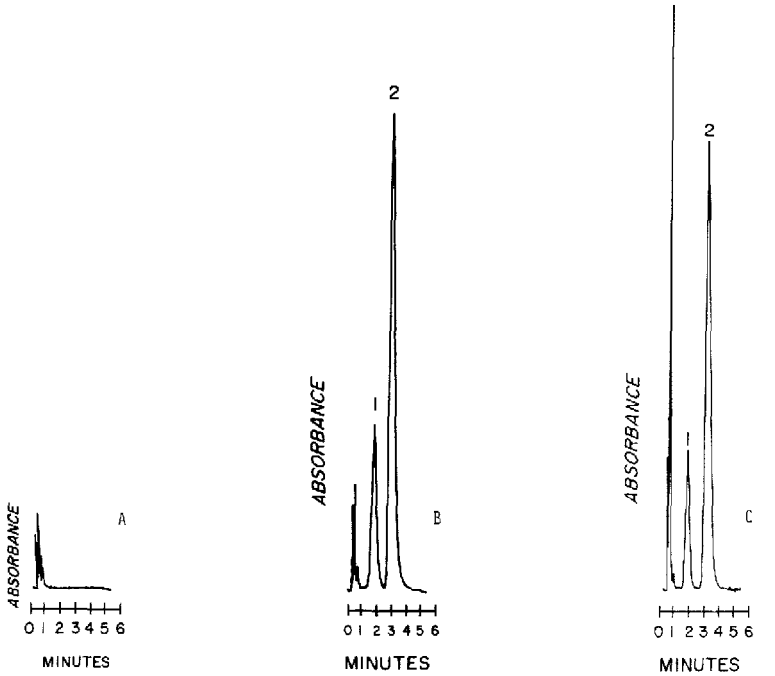

Fig 2 Chromatograms of acetonitrile extracts of (A) blank serum, (B) serum spiked with NFT and MIAA and (C) patient serum after the administration of imipenem (N-formimidoylthienamycin/cilastatin) Peaks 1 = NFT, 2 = MIAA

variation (C.V.) was 4 8 ± 2 1% for peak-area ratio and 2 2 ± 1.0% for peak-height ratio The between-day C V for peak-area ratio was 4.5%

Analysis of serum standards deproteinized by micropartition produced similar results Correlation coefficients were 0 998 and 0 999 with an average C.V of 2.9 ± 1.6% and 2.3 ± 1.5% for peak-area ratio and peak-height ratio, respectively (summarized in Table I)

TABLE I

COMPARISON OF THE LINEARITY AND REPRODUCIBILITY OF THE LOG NFT/MIAA RATIOS OBTAINED FROM HPLC ANALYSIS OF STABILIZED SERUM STANDARDS DEPROTEINIZED BY ACETONITRILE EXTRACTION OR MICROPARTITION FILTRATION

The injection volume was 8 μl, the linear range of standards was 0 8—100 μg/ml and the number of injections was 3 in both cases

Deproteinization method	Average coefficient of variation (%)		Correlation coefficient (r)	
	Peak height	Peak area	Peak height	Peak area
Acetonitrile extraction	2 2	4 8	0 995	0 996
Micropartition filtration	2 3	2 9	0 999	0 998

Sensitivity

Using an 8-μl injection volume of standard serum extract or standard serum filtrate our assay was sensitive to 0 8 μg/ml NFT. Injection volumes of 20 μl of filtrate yielded a sensitivity of 0.4 μg/ml.

Recovery

The loss of NFT during acetonitrile extraction or during filtration by the micropartition system was determined Results of the comparison of mean peak heights of serum standards with authentic standards prepared in 1 *M* MES, pH 6 5, stabilizing solution are presented in Table II NFT and MIAA recovery is greater when stabilized serum specimens are deproteinized by micropartition filtration

TABLE II

NFT AND MIAA RECOVERY FROM STABILIZED SERUM STANDARDS DEPROTEINIZED BY ACETONITRILE EXTRACTION OR MICROPARTITION FILTRATION COMPARED TO AUTHENTIC STANDARDS PREPARED IN MES BUFFER

Drug	Concentration (μg/ml)	Recovery (%)	Average C V (%)	Specimen volume (ml)	Number of injections (*n*)
Acetonitrile extraction					
NFT	100	22	1 1	0 5	3
NFT	12 5	28	3 5	0 5	3
NFT	1 6	23	4 3	0 5	3
MIAA	544	25	3 0	0 5	9
Micropartition filtration					
NFT	100	45	1 4	1 0	3
NFT	12 5	39	0 7	1 0	3
NFT	1 6	28	3 0	1 0	3
MIAA	544	39	2 6	1 0	9

DISCUSSION

Our assay for NFT in stabilized serum extract was linear with average coefficients of variation no greater than that previously reported by others (1 8—7 3%) [12, 14—16] Similarly, the correlation coefficients we observed between peak height, peak area and NFT concentration were greater than or equal to 0 995 Our observed lower limit of NFT sensitivity (0 8 μg/ml) is somewhat higher than the 0 3—0 48 μg/ml demonstrated by others However, by increasing our specimen injection volume to 20 μl our sensitivity fell within that range Gravallese et al [16] described a 10-μl injection volume in their assay, although determination of assay sensitivity was based upon data acquired using a 75-μl injection volume. Myers and Blummer [15] used a 50-μl specimen injection for their sensitivity determinations Clinically, serum NFT concentrations of less than 1 μg/ml are insignificant

The amount of NFT recovered from stabilized serum after acetonitrile extraction was less than that recovered after micropartition filtration (23 3 versus

37%). Using the latter method of deproteinization, other investigators have demonstrated drug recovery of 50 9 and 99% [15, 16]. Drug recovery may be increased by the use of a stabilized serum specimen volume of less than 1 0 ml in the micropartition system reservoir [15] Other modifications in the micropartition process may also optimize drug recovery [17]

In order to minimize NFT degradation, serum specimens should be stabilized with MES and stored at −70°C Gravallese et al. [16] demonstrated that there was no significant difference in the concentration of NFT in stabilized plasma which was stored for 42 days at −20 or −70°C They also observed a 5.9% loss of drug when a MES-buffered serum specimen was stored at 24°C for 5 h Myers and Blummer [15] demonstrated a 90% decrease in peak area in MES-buffered NFT serum specimens that were stored at −20°C and 4°C for 90 days However, specimens that were stored at −70°C for the same time period were reported as completely stable

We encountered no evidence of interfering substances in the determination of serum NFT concentration in specimens obtained during our clinical trial of imipenem [18] Cilastatin does not absorb at 300 nm and, therefore, was not an interfering substance [15, 16] Data published by Myers and Blummer [15] showed that there was no interference from fifteen drugs in common medical use Gravallese et al [16] did not encounter any interfering substances However, they identified the products of NFT degradation by preparing a 1 5 m*M* NFT solution and observing its reaction with each of three different buffers (pH 3 1, 7.25 and 10 8) The degradation of NFT and the formation of the reaction products were followed by HPLC analysis The chromatograms that were obtained showed the major metabolite, the open β-lactam ring [9], and four other reaction products as early eluting chromatographic peaks which did not interfere with the detection of NFT

In conclusion, the data compiled from our analysis of NFT serum standards deproteinized by micropartition appear analogous to the results published by other investigators despite somewhat lower drug recovery. The HPLC assay of NFT in acetonitrile-extracted stabilized serum appears as useful as the assays that utilize micropartition filtration for serum deproteinization Our assay provides an alternative method for determining NFT concentrations in pediatric human serum and does not require the use of expensive filtration systems or large specimen injection volumes The method provides results useful in determining NFT levels suitable for pharmacokinetic descriptions for pediatric patients.

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