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Study of different off-line sample processing procedures and the measurement of antibiotic and antiviral levels in human serum by high-performance liquid chromatography

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Abstract

We attempted to devise a preparation method for clinical samples that could be used for all antibiotics and antivirals. We studied thirteen antibiotics, including five penicillins, four cephalosporins, metronidazole, ofloxacin, and sulfamethoxazole and four protease inhibitors including indinavir, retonavir, nelfinavir, and sequinavir. We compared four sample preparation techniques including solvent precipitation, filtration and resin column. We employ HPLC methods based on a minimal number of columns and mobile phases. We were unable to find one sample preparation method that could be used for all antibiotics and antivirals. But, we did develop an algorithm for determining optimal processing procedures for all drugs.

Keywords: Antibiotics; Antivirals

1. Introduction

High-performance liquid chromatography (HPLC) has been associated with antibiotics since HPLC technology became available. In the pharmaceutical industry, it has been used for quality control [1] and the detection of new antimicrobials [2]. In the clinical laboratory, it has been used to predict efficacy [3] and perform routine monitoring of serum levels [4]. It has become the method against which other methods are measured [5,6], but there are problems associated with sample preparation [7–10].

Antibiotics bind to the filters and resins used to prepare biological samples for analysis as well as to serum and urine components [11,12]. HPLC sample preparation techniques can influence subsequent antibiotic concentration determination [12].

This study was undertaken to determine if a single, rapid processing method could be devised for clinically relevant antibiotics and antivirals. An ideal method would be rapid, would require few steps, and would be useful for different classes of drugs. This study examined four sample preparation techniques for 13 different antibiotics and four protease inhibitors. Antibiotics included five penicillins, four

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cephalosporins, metronidazole, ofloxacin, and sulfamethoxazole. Protease inhibitors included indinavir, retonavir, nelfinavir, and sequinavir. Sample preparation methods included solvent precipitation, two types of filters, and a resin column. We discovered no single optimal processing method, but we do present an algorithm useful when quantitation of antibiotics or antivirals in serum is necessary.

2. Experimental

2.1. Chemicals and test compounds

Spectrophotometric-grade methanol and acetonitrile were purchased from Curtin Matheson Scientific (Houston, TX, USA). All water was doubly distilled and produced in the laboratory. Chemicals were purchased from Sigma (St. Louis, MO, USA). Normal human serum was purchased from Flow Labs. (McLean, VA, USA) and had 4.2 g per deciliter albumin.

Ampicillin and nafcillin were purchased from Marsam (Cherry Hill, NJ, USA). Cefazolin, methcillin and oxacillin were purchased from Apothecon (Cherry Hill, NJ, USA). Cefotaxime, and desacetyl cefotaxime were a gift from Hoechst-Roussel (Somerville, NJ, USA). Cefotetan was purchased from Zeneca (Wilmington, DE, USA). Ceftazidime was purchased from Glaxo Wellcome (Research Triangle Park, NC, USA). Ceftriaxone was purchased from Roche (Nutley, NJ, USA). Indinavir was a gift from Merck (Rahway, NJ, USA). Metronidazole was purchased from Searle (Chicago, IL, USA). Nelfinavir was a gift from Agouron (Garden City, UK). Ofloxacin was a gift from McNeil-PPC (Fort Washington, PA, USA). Ritonavir was a gift from Abbott Labs. (Chicago, IL, USA). Sequinavir was a gift from Roche (Welwyn Garden City, UK). Sulfamethoxazole was purchased from Sigma. Ticarcillin was purchased from Smith-Kline Beecham (Philadelphia, PA, USA).

2.2. HPLC apparatus and conditions

Chromatography was performed with a Beckman Model 110A pump (Beckman Instrument, Brea, CA, USA), a reversed-phase Econosphere C_{18} 5 μm column (Alltech, Deerfield, IL, USA) for antibiotics and a Zorbax SB-C18 5 μm column (Hewlett-Packard, Wilmington, DE, USA) for protease inhibitors. The injector was an Altex Model 210 equipped with a 20 μl loop. The detector was a Hitachi L-4250 UV–Vis detector with a 5 mm flow cell (Hitachi, San Jose, CA, USA). The data handling system was a Perkin-Elmer Nelson Model 1020 operating on a dedicated personal computer. Flow rate was 1 ml/min and absorbance units full scale (aufs) was 0.01. Mobile phase, retention times (t_R), and detection wavelengths are given in Table 1.

2.3. Sample preparation

Ultrafree-MC, low binding regenerated cellulose filter with 10 000 nominal molecular mass limit (NMWL), Ultrafree Biomax 5 K membrane filter with 5000 NMWL and Ultrafree Biomax 10 K membrane filter with 10 000 NMWL were all purchased from Millipore (Bedford, MA, USA). Oasis extraction cartridges were purchased or were a gift from Waters (Milford, MA, USA).

Methanol precipitated samples were prepared by adding 0.1 ml of serum with drug to 0.9 ml cold methanol. The sample was then centrifuged at 2500 *g* in a swinging bucket centrifuge for 5 min to precipitate protein.

Filtered samples were prepared by placing 0.2 ml serum with drug on the filter and centrifuging at full speed for 15 to 30 min in a bench top microfuge Capsule HF-120 purchased from Tomy Seiko (Tokyo, Japan).

Oasis columns were washed with 1 ml volumes of methanol then water before the sample (drug dissolved in either water or serum) was applied to the top of the column. After the sample drained, the column was washed with methanol-water (5:95) and the analyte eluted with 100% methanol and collected. Protease inhibitors in clinical samples were concentrated by drying the 100% methanol eluate then resuspending in HPLC buffer at 1/10 or 1/20 the original volume for analysis. Antibiotics were assayed without concentration since their serum levels are so much higher.

2.4. Data analysis

Area under the peak was measured and stored by the data handling system. Parameters describing the

Table 1
Mobile phases, retention times and detection wavelengths of drugs studied

		Mobile phase, MeOH–aqueous (%)*	t_R (min)	Wavelength (nm)
Antibiotics				
Penicillins	Ampicillin	25:75	12.5	230
	Methcillin	35:65	8	230
	Nafcillin	50:50	7	230
	Oxacillin	45:55	6	230
	Ticarcillin	25:75	6.5	230
Cephalosporins	Cefazolin	25:75	6	250
	Cefotaxime	20:80	6	250
	desacetyl	20:80	7	250
	Cefotetan	15:85	3.2	250
	Ceftazidime	25:75	5.5	250
	Ceftriaxone	[a]	4.5	280
Metronidazole		25:75	6	313
Ofloxacin		[b]	4.3	288
Sulfamethoxazole		45:55	5.4	260
Protease inhibitors		Acetonitrile–aqueous**		
Indinavir		35:65	6.1	210
Nelfinavir		60:40	4.9	210
Ritonavir		40:60	16.5	239
Sequinavir		40:60	23	239

*Antibiotic aqueous buffer: 0.1 *M* sodium acetate, pH 4.8 with glacial acetic acid.

**Protease inhibitor aqueous buffer: 25 m*M* sodium acetate and 25 m*M* hexane-1-sulfonic acid, pH 6.0 with 37% HCl.

[a] A 600-ml volume of acetonitrile + 10 ml 1 *M* phosphate buffer, pH 7.0 + 3 g hexadecyltrimethylammoniumbromide + doubly distilled water to 1 l.

[b] A 150 ml volume of acetonitrile + 10 ml potassium phosphate buffer, pH 7.0 (13.61 g KH_2PO_4/100 ml, 17.12 g K_2HPO_4/100 ml. Mix for pH 3.4) + 3 g hexadecyltrimethylammonium bromide + doubly distilled water to make 1 l.

standard curve were calculated with the Perkin-Elmer Nelson Model 1020 system set to include the origin and three experimentally determined points assayed in triplicate. Response factors for each antibiotic and protease inhibitor were determined from standard curves generated three times in triplicate assays from manufacturer's supplied standard powder stock solution at 1 mg/ml measured with a five place balance (Mettler Electronic, Anaheim, CA, USA). The stock solution, optimally dissolved according to manufacturer's directions, was diluted to 10 and 5 μg/ml. Standard curves were constructed from injections of 25, 50, and 100 ng. Injections of 200 ng were used to determine if linearity extended well past the imposed upper limit of 100 ng. The limit of quantitation (LOQ) for each assay was based upon guidelines from the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals in Human Use [13]. The LOQ was defined as the response factor multiplied by 10 standard deviations of noise detected at the appropriate wavelength with the appropriate buffer flowing through the system. Recovery of each antibiotic from water and normal human serum and protease inhibitor from water and normal human serum was determined by triplicate, independent assays.

All statistical analysis was performed by the Perkin-Elmer Nelson data handling system or Excel statistical tools.

3. Results and discussion

Table 1 lists the mobile phase compositions, the retention times and the wavelength determined to be optimal for our laboratory environment.

Table 2 lists response factor as ng per 10^5 area units full scale, r^2 of the standard curve, and limit of quantitation as ng per ml for all drugs included in this study. The maximum response factor was ampicillin at 91.2 ng/10^5 aufs meaning 91.2 ng of

Table 2
Response factor, correlation coefficient and limit of quantitation of drugs

		Response factor ($ng/\cdot 10^5$)	r^2	LOQ[a] (ng/ml)
Antibiotics				
Penicillins	Ampicillin	91.2	0.967	20
	Methcillin	61.1	0.986	22
	Nafcillin	3.2	0.994	12
	Oxacillin	25	0.985	20
	Ticarcillin	37.96	0.9905	15
Cephalosporins	Cefazolin	2.5	0.9654	0.24
	Cefotaxime	10.35	0.9696	7.7
	desacetyl	10.5	0.9676	4
	Cefotetan	31.2	0.991	4
	Ceftazidime	23	0.997	10
	Ceftriaxone	10.3	0.965	5
Metronidazole		7.7	0.995	0.13
Ofloxacin		6.1	0.934	20
Sulfamethoxazole		7.7	0.969	0.7
Protease inhibitors				
Indinavir		7.4	0.99	7 pg/ml
Nelfinavir		8.06	0.99	1.7 pg/ml
Ritonavir		39.7	0.99	38.3 pg/ml
Sequinavir		7.9	0.98	3.6 pg/ml

[a] Limit of quantitation.

ampicillin was needed to give a response of $1\cdot 10^5$ absorbance units. Minimum response factor was cefazolin at 2.5 $ng/10^5$ aufs meaning only 2.5 ng of cefazolin was needed to give a response of $1\cdot 10^5$absorbance units. These differences are reflected in the limit of quantitation calculated for each drug.

Table 3 shows the percent of antibiotic recovered after processing an aqueous preparation through each of the filters or the Oasis extraction cartridge. Inversely it reflects the percent of antibiotic bound to each filter or cartridge. The Millipore Ultrafree-MC (MC) bound less of the studied penicillins (average 20%) than cephalosporins (average 28%). Ultrafree Biomax 5 K and 10 K bound less of the studied cephalosporins (average 4%) than penicillins (average 29%). The Oasis cartridge varied in the amount of penicillins and cephalosporins bound.

Table 4 shows the percent of antibiotic recovered when normal human serum containing antibiotic was processed by methanol precipitation of serum proteins, and filtration through MC, 5 K or 10 K filters, and Oasis columns. Methanol precipitation of the serum sample yielded nearly complete recovery of penicillins, and lower recoveries of cephalosporins. Methcillin recovery by methanol precipitation was greater than 100% reflecting a co-eluting contaminating substance. Recovery after filtration through any filter and the Oasis column was dependent on binding to both serum proteins and each filter membrane or column resin.

All drugs except cefotetan with MC filters and ampicillin and oxacillin with 5/10 K filters showed lower recovery when dissolved in serum than in water reflecting binding to serum proteins [11,14].

Some type of sample preparation is mandatory for HPLC analysis of drugs in serum. Serum cannot be injected directly onto HPLC columns; the high protein concentrations lead quickly to column degradation and plugging. Solvent precipitation using cold methanol separates antibiotic from serum proteins by precipitating the proteins. This is not 100% effective, but protein levels are reduced enough that the supernatant can be repeatedly injected onto the HPLC column before a guard column has to be replaced. Filtration of a serum sample also removes proteins. This method is no more effective at removing proteins than solvent precipitation, but again is adequate. The optimal method to remove serum

Table 3
Percentage of drug dissolved in water recovered from each system

		MC filter	5 K filter	10 K filter	Oasis column
Antibiotics					
Penicillins	Ampicillin	75.9±3.8	72.2±2.0	67.2±1.9	91.3±0.9
	Methcillin	94.7±2.6	84.3±6.2	82.5±2.2	76.4±6.2
	Nafcillin	63.3±1.5	37.1±1.1	50.6±0.6	83.8±0.4
	Oxacillin	75.7±1.7	73.4±1.3	73.3±1.3	100.1±2.4
	Ticarcillin	94.6±6.8	98.9±1.9	N.D.[a]	31.1±3.0
Cephalosporins	Cefazolin	57.4±4.0	91.4±3.0	97.8±3.1	71.6±11.2
	Cefotaxime	66.6±0.9	92.9±1.1	100.5±2.0	72.4±9.1
	desacetyl	69.0±3.6	90.8±2.0	96.1±4.2	N.D.
	Cefotetan	41.7±3.3	N.D.	101.7±5.0	4.4±0.3
	Ceftazidime	98.7±1.9	91.0±3.0	103.9±2.6	44.7±0.7
	Ceftriaxone	98.3±1.8	98.0±2.4	101.6±2.5	24.9±1.8
Metronidazole		95.5±6.8	70.4±0.9	53.9±1.7	100±3.4
Ofloxacin		93.9±6.7	81.0±2.3	N.D.	118±7.1
Sulfamethoxazole		76.2±4.4	72.4±6.8	N.D.	91.4±0.5
Protease inhibitors					
Indinavir					92.4±11.8
Nelfinavir					97.1±2.2
Ritonavir					93.0±11.2
Sequinavir					96.0±8.0

[a] Not done.

Table 4
Percentage of drug dissolved in normal human serum recovered from each system

		Methanol precipitation	MC filter	5/10 K filters	Oasis column
Antibiotics					
Penicillins	Ampicillin	104.6±21.6	64.6±1.9	69.7±2.0	37.7±1.2
	Methcillin	400%	39.8±2.1	38.6±2.8	11.1±1.4
	Nafcillin	93.9±4.2	5.4±0.4	2.4±0.1	11.3±0.1
	Oxacillin	104.1±2.3	15.3±0.3	75.0±0.9	100%
	Ticarcillin	71.9±3.3	54.3±2.9	55.4±9.3	31.8±1.5
Cephalosporins	Cefazolin	43.0±0.2	15.8±2.0	2.6±0.4	64.3±3.7
	Cefotaxime	61.2±3.4	50.2±0.4	62.4±2.4	77.6±3.1
	Cefotetan	107.2±10.5	48.4±2.4	46.0±1.6	36.9±3.3
	Ceftazidime	100±12	33.1±9.7	53.5±3.5	25.9±0.6
	Ceftriaxone	69.0±2.2	6.4±0.2	50.5±1.6	18.0±0.35
Metronidazole		82.3±0.5	85.6±2.9	53.6±2.5	140%
Ofloxacin		BDL*	9.7±0.1	11.5±0.5	63.6±0.9
Sulfamethoxazole		92.0±9.9	43.7±0.6	8.7±0.8	77.3±3.6
Protease inhibitors					
Indinavir					96.2±8.5
Nelfinavir					25.2±2.8
Ritonavir					32.5±1.9
Sequinavir					58.9±11.4

*Below detectable limits.

proteins is differential extraction of the antibiotic and removes more protein than any other procedure. We have not included this procedure in this study because it is much more time consuming and cumbersome—not well suited for clinical usage. The final method evaluated used a resin column. In this procedure the analyte of interest was theoretically held on the column, washed, and then eluted for injection onto the HPLC column. This method effectively removed protein from the sample, but could not be used for all the antibiotics in this study since the column did not retain some antibiotics. It was the only method studied that could be used for protease inhibitor quantitation.

Recovery of antibiotic from serum depended upon the extent to which the antibiotic was bound to serum components and/or filters or resins used to prepare the serum for analysis. According to Craig and Suh [11] ionic (or electrostatic) and hydrophilic bonds are the primary bonds through which drugs bind to serum and tissue components. Albumin with approximately 100 acidic groups, 86 basic groups and 56% hydrophobic residues is thought to be the predominant drug binding protein. But other serum proteins bind other drugs. For example, α-, β-, and γ-globulins bind erythromycin, fibrinogen binds rifampin and lipoproteins bind tetracycline. The methods used in this study help elucidate binding of the antibiotics studied. Results for nafcillin, ceftazidime and ceftriaxone illustrate three slightly different scenerios.

Nafcillin was recovered 95% by methanol precipitation, 60% from MC filters when dissolved in water, 5% when dissolved in serum, 40–50% from 5/10 K filters dissolved in water, and 5% when dissolved in serum. According to previously published equilibrium dialysis data nafcillin is approximately 90% bound to serum proteins [11]. Methanol disrupted that binding and resulted in recovery of nearly 100% of total nafcillin present in the serum. Filtering the sample did not disrupt binding and free nafcillin was measured.

Ceftazidime was recovered 100% by methanol precipitation, 99% from MC filters when dissolved in water, 35% when dissolved in serum, 100% from 5/10 K filters when dissolved in water and 50% when dissolved in serum. Reported ceftazidime binding levels range from <10% [14] to 17% [11]. Our data implied greater binding to serum proteins, in the 50 to 65% range. Again methanol precipitation disrupted any binding and yielded total ceftazidime levels. Filtration did not disrupt binding and yielded free ceftazidime levels.

Ceftriaxone binds to two sites on human albumin: one a high affinity, low capacity site, the second a low affinity, high capacity site [15–17]. Methanol precipitation disrupted the low affinity, high capacity site and recoveries approached 100%—depending upon the amount of drug bound to the high affinity, low capacity site. Filtration did not disrupt the low affinity, high capacity binding and recovery percentages were low and represented only free drug.

Table 5 shows why Oasis columns were useful for only three antibiotics: oxacillin, metronidazole, and ofloxacin. Oasis column fractions were individually assayed for most antibiotics and all antivirals. The drugs were dissolved in water for the study. Oxacillin and metronidazole eluted in only the final 100% methanol wash, but over half of ticarcillin, cefotetan and ceftriaxone eluted in the sample application volume—before any washes.

Oasis columns were invaluable for protease inhibitors. Greater than 90% of the protease inhibitors eluted in the final methanol wash and an additional 100% methanol wash did not recover significantly more. Data not shown found a correlation coefficient of 0.98 for indinavir measured with Oasis column technology compared to manufacturer's suggested differential extraction procedure. The concentration capability of the Oasis column allowed measurements in the ng/ml range. This was not possible with solvent precipitation or filtration.

The objective of a single extraction procedure for antibiotic serum levels remains elusive. We have developed a sample preparation algorithm for assay of serum antibiotic levels:

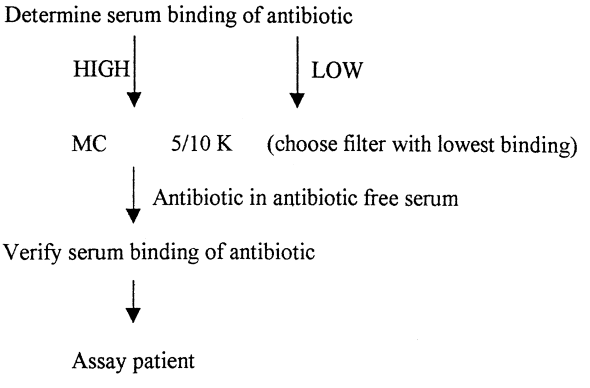

Table 5
Percentage distribution of antibiotics dissolved in water in each Oasis fraction

		Sample eluate	5% methanol wash	100% methanol wash	
Antibiotics					
Penicillins	Ampicillin	BDL*	8.7	91.3	
	Methcillin	18.1	5.4	76.4	
	Nafcillin	14.7	1.5	83.8	
	Oxacillin	BDL	BDL	100	
	Ticarcillin	56.1	12.7	31.1	
Cephalosporins	Cefazolin	23.3	5.2	71.6	
	Cefotaxime	20.3	7.3	72.4	
	Cefotetan	85.6	10	4.4	
	Ceftazidime	37.7	17.6	44.7	
	Ceftriaxone	64.2	10.9	24.9	
Metronidazole		BDL	BDL	100	
Ofloxacin		0.9	0.55	118	
Sulfamethoxazole		1.8	6.8	91.4	
Protease inhibitors					Second 100% wash
Indinavir		1.3	BDL	92.4	6.3
Nelfinavir		BDL	BDL	97.1	2.9
Ritonavir		BDL	BDL	93	7
Sequinavir		1.6	BDL	95.9	2.4

*Below detectable limits.

Solvent precipitation can be used to quickly assay either total antibiotic present in a serum, or to quantitate an antibiotic with very low serum binding. An antibiotic with higher serum protein binding will have to be assayed by either filtration or column methods. Determine the optimal filter or resin by measuring the binding of the antibiotic dissolved in water to the filter or resin. Choose the material with the lowest binding or highest recovery and assay an antibiotic free serum to which the antibiotic of interest was added at known concentration. Assay the serum submitted for analysis.

Oasis columns are the only method to use for protease inhibitors, but they have to be evaluated on a case by case basis for antibiotics since no consistent pattern was found. The column would be useful for oxacillin, metronidazole or ofloxacin but not for any other antibiotic studied.

The addition of an internal standard of known concentration is a well-accepted method used to resolve recovery questions. The assumption on which this principle operates is one of equivalent recoveries of members of the same class or molecules of similar chemical structure to the antibiotic of interest. Obviously care must be taken to match the internal standard to the analyte of interest for binding to serum proteins as well as to filters or resins used in sample preparation.

4. Conclusions

We have not determined a single preparation procedure, but we present a logical framework for the determination of clinical antibiotic serum levels. For antibiotics the initial step should be a determination of the serum binding of the antibiotic of interest by reference to literature sources. For antibiotics with very low binding, or for determination of total antibiotic present in the serum, solvent precipitation is the optimal sample preparation procedure. For more highly protein bound antibiotics the binding of the antibiotic to the filters must be determined. It is reasonable to start with the Ultrafree MC filter for penicillins and with an Ultrafree Biomax filter for cephalosporins since generally these classes of antibiotics bind least to the respective filter membranes. The initial determination of binding to the filter is done with the antibiotic of interest dissolved in water. The addition of a spiked serum sample verifies optimal processing before performing clinical assays.

Protease inhibitors can only be quantitated with Oasis columns. Their total serum concentrations are too low to be determined by filtration or solvent precipitation and UV detection. The concentration of the sample possible with Oasis columns allows accurate quantitation of ng/ml levels.

The addition of an internal standard of known concentration must be made with care to match the analyte binding to serum proteins and filters and resins used for sample preparation.

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