Application of an On-Line Restricted Access Material (RAM)/Liquid Chromatography/Tandem Mass Spectrometry Method for the Rapid Determination of Veterinary Drug Residues in Fish

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ABSTRACT

Current veterinary drug residue analytical methods using mass spectrometry detection often require time-consuming sample clean-up steps before sample analysis can occur. An on-line technique using a "Restricted Access Material" or "RAM" column was evaluated to determine the feasibility of achieving sample clean-up in a different, simpler, and much quicker fashion. On-line RAM procedures have been widely used for drug analysis in the last twenty years, but are applied almost exclusively to biological fluids. The LIB procedure described here used a RAM process and applied it to tilapia, catfish and salmon muscle tissue for a quantitative multi-analyte veterinary drug residue method. A total of 38 of 62 "test compounds", representing a wide variety of veterinary drug classes, were recovered at the level of interest in this initial feasibility study. The RAM approach had both advantages and some drawbacks for use as a multi-residue method. The methodology was extremely simple, rapid, and very rugged with the RAM column still maintaining good performance even after several hundred injections. Many compounds from the test mixture had good recovery, sensitivity, and reproducibility. However, some analytes were not recovered using the procedure, mostly small and very polar analytes, and it is also difficult to predict why other analytes were not successful with the RAM approach used. Overall, this technique has potential to be useful in veterinary drug residue analysis.

INTRODUCTION

There are many different published methods describing the analysis of veterinary drug residues in tissue using mass spectrometry. Most of these procedures share some common features. First, most multi-residue methods use an organic extraction solvent to extract the analytes from the tissue. The organic solvent may be modified with small amounts of water or acid to allow for the extraction of the widest range of both polar and non-polar analytes. Second, after extraction, the sample extract usually undergoes a clean-up step. The two most common clean-up procedures for tissue extracts are Solid Phase Extraction (SPE) and Dispersive Solid Phase Extraction (dSPE). The SPE technique involves passing the extract through a packed cartridge containing a solid sorbent while

the dSPE procedure involves blending the extract with a solid sorbent, centrifuging, and then decanting off the remaining extraction solution. The goal of the second step is to primarily remove phospholipids and other interferences (which are known to cause MS signal suppression) from the matrix in the sample extract. Third, the cleaned-up extract is often evaporated and reconstituted with a mostly aqueous solution before final injection into the LC/MS system. The reason for this step is twofold. Almost all LC chromatographic systems used for a multi-residue separation involving polar and non-polar analytes use reverse-phase chromatography. In reverse phase-systems, the initial gradient usually starts out with a mobile phase of primarily water (or acidified water) and then more non-polar compounds are successively eluted from the HPLC column by increasing the organic component in the mobile phase over time. This means, however, that the composition of the final liquid in the sample injection vial must also be primarily aqueous (instead of organic). If it isn't, extremely poor chromatography occurs (i.e. peak broadening and splitting) on a reverse-phase system, especially for more polar analytes. Evaporation and reconstitution of the organic extract with an aqueous solution corrects this issue. In addition, if the initial volume of extract is reduced by evaporation and is reconstituted with a much smaller volume, the analytes are concentrated, improving detector response. The initial organic sample extract also can be simply further diluted with water (avoiding the evaporation step) to achieve reverse-phase compatibility, but this usually compromises detection sensitivity. Since many veterinary drug residues need to be detected at low ppb levels, this often makes further dilution of the initial extract impractical.

A RAM approach would ideally eliminate the last two time-consuming steps (clean-up and evaporation/reconstitution) in a multi-analyte method. Using RAM, the tissue is still extracted in a mostly organic solvent, but this extract is then directly injected into a set-up consisting of a separate RAM column in conjunction with an HPLC analytical column.

The RAM column consists of particles packed inside a metal cartridge and holder with HPLC compatible fittings. An illustration of the RAM column particle used in this LIB is shown in Figure 1. Two different chromatographic processes (size exclusion and partition) occur within the RAM column. First, the small pore size of the RAM particles prevents bigger molecules >15 kDa (1) from accessing the pores (are "restricted from") the pores. The outer surface of the spherical RAM particle has bonded diol groups which do not adsorb the bigger proteins or other hydrophilic cellular molecules and salts which will then be quickly washed away to waste in the initial highly aqueous mobile phase. Second, the much smaller organic target analytes should diffuse into the smaller pores and be retained and pre-concentrated by the bonded C18 groups inside the pores. This specific type of RAM particle is referred to as an ADS ("Alkyl-Diol") system and is the type most widely used (2).

The analytes retained inside the pores are then released from the RAM column by increasing the organic content of the mobile phase. The eluted compounds are then directed to the LC analytical column and MS detector. To accomplish this process on-line, two separate binary pump systems and a switching valve are needed as shown in Figure 2. Both sets of binary pumps have their own separate gradient profiles throughout the run. This allows conditioning, washing and equilibrating of both the RAM column and LC analytical column simultaneously. The left side of Figure 2 shows the initial flow diagram where the sample extract is injected ("loading position") onto the RAM column. The initial RAM mobile phase is mostly aqueous at this point, allowing for the

unwanted non-adsorbed proteins to pass through the column and into waste. The right side of Figure 2 shows the transfer position whereby the 6-port switching valve (SV) is rotated 60 degrees to now couple the RAM column to the analytical column. The SV operates at a set time, after the proteins have finished passing through the RAM column. It is important to note that the flow direction has now changed, resulting in a backflush of the original head of the RAM column, which causes additional concentration of the retained analytes (2). The secondary pump system then increases the organic content of the mobile phase and the analytes are eluted on to the analytical column. Because of the focusing effect of the analytes on the head of the RAM column, it is possible to use higher injection volumes without affecting peak shape or overloading the analytical column capacity. For this LIB, 75 µL of filtered extract was injected onto the RAM column. The large injection volume increased method sensitivity, eliminating the need to concentrate analytes in the extract by evaporation and reconstitution.

EXPERIMENTAL

Extraction

1. Weigh 2.0 ± .05 g of homogenized tissue into a 50 mL polypropylene tube.
2. Add spiking standard mixtures as appropriate. (Spiking standards are as per LIB 4615-see later discussion section: Compounds and Testing Levels and also Table 1).
3. Add 6 mL of extraction solution. The extraction solution is also made per LIB 4615. The extraction solution consists of 0.2% p-toluenesulfonic acid monohydrate (w/v) and 2% glacial acetic acid (v:v) in 75% acetonitrile (ACN).
4. Vortex for 30 minutes (Fisher Multi-tube vortexer, setting speed 2500 rpm).
5. Centrifuge the tubes for 7 minutes at 4° C at 10,000 rpm or 17,000 RCF (g).
6. Filter a portion of the extract through a 0.2 µm nylon syringe filter into a limited volume HPLC vial.

Equipment and Reagents

1. Centrifuge: Thermo Scientific Sorvall RC +6 programmable refrigerated centrifuge capable of speeds of 13,000 rpm or 28,900 RCF (g) or equivalent.
2. Mechanical shaker: multi-tube vortex mixer (Part # 02-215-450, Fisher Scientific, Pittsburgh, PA) or equivalent.
3. Vortexer: Fisher Scientific multi-tube 6, operated at a speed setting of 2500 or equivalent.
4. ρ-Toluenesulfonic acid monohydrate: ACS reagent grade (>98.5%) from Sigma Aldrich.
5. ACS grade glacial acetic acid (EMD chemicals or equivalent).

6. Solvents: methanol, acetonitrile, formic acid and water were all Optima LC/MS grade from Fisher Chemical.

7. Nylon syringe filters: 13 mm, 0.2 µm PN Z259942 were from Sigma.

Instrumentation

LC separation was performed using an Agilent Eclipse XDB C18 (4.6 x 150 mm, 5 µm) reversed-phase column. The guard column (inserted just before the analytical column) was a Phenomenex C18 4 x 3.00mm cartridge with associated holder PN AJO-4287. The RAM column was a LiChrospher RP-18 ADS 25 x 4 mm cartridge with an associated LiChro-CART housing PN 1.50947.0001 from EMD Chemicals. Table 2 shows the two independent mobile phase gradients for the RAM column and the LC analytical column. Mobile phase A was 0.1% formic acid. Mobile phase B was acetonitrile for both sets of pumps and columns.

LC/MS Parameters

The LC system was an Agilent 1200 dual binary pump with degasser and column heater. The auto sampler was a Leap Technologies Pal HTC Pal. The injection volume was 75 µL, and the column oven temperature was 50º C.

The MS was an ABSciex 5500 QTrap® with Analyst® software version 1.6. Electrospray ionization was used in the positive mode operated at 700º C source temperature and 5500V. The gases had the following pressures: curtain gas, collision gas were 25 and 45 psi N_2 respectively. Gas supply 1 was 60 psi zero air, gas supply 2 was 50 psi zero air. Declustering, entrance and exit potentials, and collision energies of MRM product ions were optimized by infusion of standards-values used were from (3). Three product ions were monitored for each analyte. The dwell time for all transitions was 75 msec. Resolutions for Q1 and Q3 were each set at unit mass resolution.

RESULTS AND DISCUSSION

The phrase “Restricted Access Material” was introduced in 1991 (4). Almost all later published RAM applications (1,2,4) involve analyzing various drugs or their metabolites in biological fluids such as plasma, serum, urine, saliva, or various cell cultures. A comprehensive review (4) discusses the use of different RAM supports applied to the analysis of these matrices. There are only a few RAM applications for food, mostly for liquids such as fruit juice and milk. The primary goal of the research here was to investigate the initial feasibility of a RAM approach to solid fish tissue matrices, specifically geared toward the applicability for a multi-residue method.

When adapting a RAM approach for a tissue extract, two of the most important variables to consider are the RAM column flow rate, and rotor valve switching times (see Figure 2). Researchers have reported (1,4) that choosing the flow rate of the mobile phase through the RAM column is important. High flow rates cause turbulent flow (as opposed to laminar) resulting in eddy strengths that extricate the large molecules that would otherwise block smaller pores on the RAM column. This turbulent flow rate greatly increases the lifetime of the RAM column (4). A

flow rate of 1 mL/min or higher is needed to achieve turbulent flow with a 25µm RAM particle (1) and was used in this LIB. The larger size of the particles in the RAM column are designed to allow for higher flow rates without causing pump high back pressure. However, flow rates higher than 1 mL/min can also make analyte breakthrough more likely to occur for polar analytes using an ADS RAM column.

The timing of the switching valve (labeled SV in Figure 2) is another critical parameter and two separate switching times occur during the chromatographic run. First, the RAM column needs to be directed to waste long enough for the non-retained proteins to be discarded before the SV is rotated over to the analytical column. But too long a time at this point may cause some analytes to elute from the RAM cartridge, and then be diverted to waste and lost. Published research (5-8) for applications in biological fluids, using a C18 ADS LiChrospher cartridge and similar mobile phases as used in this LIB, often set the load time at 8-10 minutes. (The pump and switching valve set up for the load time is shown on the left side of Figure 2). For this reason, a load time of 8 minutes was used for our fish matrix as well. The second important time to set is how long the RAM column and the LC analytical column are together in the same flow path (as shown in the right side of Figure 2). Back-flushing the analytes off the front of the RAM column onto the HPLC column occurs at this point. At some point the RAM column must be switched back to waste to wash it with stronger solvent and then return it to mostly aqueous composition to make it ready for the next injection (simultaneously, the HPLC pump system will begin its own gradient for analyte separation on the LC column). However, it is important that the RAM column not be switched away from the LC column too early, before all the analytes have had a chance to elute off the RAM column into the LC column. The maximum time the two columns would need to be connected is the time it takes the latest eluting analyte on the RAM column to exit the cartridge. The latest eluting test compound on the LC analytical column was dicloxacillin at 20.3 minutes. The right side of the flow path in Figure 2 was switched back to the load position at 17 minutes to re-equilibrate the RAM column for the next injection. (At 17 minutes dicloxacillin has eluted from the RAM column, even though it doesn't elute from the analytical column for another three minutes).

Because it was not known how rugged the ADS RAM column would be with tissue extracts or how effective they would be in removing tissue matrix compounds, a small C18 guard column was installed in front of the analytical column for protection. The guard column would need to be changed after a few hundred injections (after observing a slight increase in pressure) but the C18 RAM column itself proved to be remarkably rugged, easily lasting several hundred injections without seeming to compromise performance. This is important as a RAM column is generally more expensive than a typical HPLC column.

Compounds and Target Testing Levels

The veterinary drugs chosen (see Table 1) as test compounds for RAM analysis were those of LIB 4615 (9). LIB 4615 describes the development of an extraction and clean-up procedure for veterinary drug residues in fish using a new specialty phospholipid SPE cartridge. The LIB 4615 extraction method was then also used with high-resolution Orbitrap mass spectrometry (10). All stock standards were made per LIB 4615. The "1X" or Target Testing Level (TTL) spiking standards used for the RAM procedure described here were also prepared per LIB 4615. Since the

tissue extraction solution of LIB 4615 extracts from fish all the compounds listed in Table 1 to some extent, the same extractant solution was used here for the RAM approach as well. The 1X level spikes and extracted matrix standards were also prepared using 2.0 g of negative control tissue as in LIB 4615. A five-point matrix-extracted standard curve (from 1X to 5X) was injected with each analytical batch and used for quantitation.

Ten spikes each were run at 1X, 2X and 3X levels for each matrix of tilapia, salmon, and catfish. Tilapia has the least amount of fat, while salmon has the most, and these different fish matrices should provide a good comparison test for the C18 RAM column performance. Table 3 summarizes the results for the tilapia spikes. Overall, 38 of 62 compounds from the target list were recovered by the RAM procedure described here (see Tables 1 and 3).

The target compounds that were not recovered can be grouped into three categories. First, a C18 RAM column does not seem to retain small very polar analytes with the parameters used here. These molecules might not have enough non-polar portions to be adsorbed inside the reverse-phase particle pores during the loading phase when the mobile phase is 98% aqueous. This has been previously reported (3) with ADS RAM columns. This is probably why analytes such as SAA, SDZ, MNZ, AMOX and FFA were not recovered. Penillic Acid, a breakdown product of PEN G, does not occur at a relevant amount (<2000 area counts) in any matrix standard injection. (Because the acidic sample extract is never heated in the RAM procedure, PEN G does not seem to degrade appreciably). Second, some of the target analytes are known (11) to be difficult to analyze in any multi-residue method. These include the triphenylmethane dyes, and their less polar leuco metabolites LCV and LMG, which could not be detected in the RAM method. The chromic dye forms BG, CV, and MG had variable recoveries and their matrix standard curves (especially BG) were less linear in response as well. The very non-polar avermectins IVER and DOR also did not work with the RAM procedure, although EMA worked somewhat but with variable recoveries and some non-linearity was demonstrated. Avermectins are also difficult to incorporate into multi-residue methods (11) as they tend to form various adducts and are often analyzed by stand-alone methods. Third, there are some target analytes for which there is no obvious reason why they were not recovered, as they have some non-polarity and it would be predicted that they would retain inside the C18 particle pores. It may be that pH plays a role. It is possible some of the analytes have pKa's which cause them to be partially charged at the pH of the non-buffered mobile phase used when loading the RAM column, causing them to be more soluble in the initial loading aqueous mobile phase and therefore lost to waste.

However, the procedure seemed to work well for many analytes listed in Table 3, with both good linearity and recovery. Although a less efficient 5 µm analytical column was used, the peak widths for almost all analytes was less than 0.3 min at half maximum, indicating that injecting 75µL of the extract onto the RAM column with subsequent elution did not appreciably contribute to band broadening. The peak widths of the tetracyclines were sometimes over 0.3 min, but these compounds are known to be difficult to chromatograph using MS-compatible mobile phases. No significant carryover to a blank injection after a standard injection was noticed except for CV which had as high as 40% carryover. This compound, however, is notorious for injector carryover (11) and this issue is not likely to be caused by the RAM process. The tetracyclines sometimes had around 10% carryover, but this is probably due to the high signal response of these analytes as their 1X level is set at the high level of 75 ppb. Two separate samples of catfish tissues (dosed

with enrofloxacin) were previously analyzed in the Denver Laboratory using a LC/MS Orbitrap screening procedure (10). These same samples were also analyzed by the quantitative RAM procedure described here and the two methods gave similar results for both enrofloxacin and ciprofloxacin.

It has been reported (6) that even after RAM column cleanup, some matrix effects or MS signal suppression can still occur. This was also noticed in the RAM procedure used here (by comparing a 1X matrix standard MS signal response to that of an equivalent 1X solvent standard). Many analytes in the RAM tilapia matrix standard had around 50% response compared to the solvent standard. For the quinolones and fluoroquinolones, there was no appreciable matrix suppression in the two different standards. Tetracyclines, emamectin and the triphenylmethane dyes had more severe matrix effects. This requires that matrix-matching standard curves be used to make the RAM procedure quantitative, but with the simple extraction, this is more easily accomplished than with most quantitative residue methods.

Additional ADS RAM columns having C4 or C8 (instead of C18) bonded inside the pores were also evaluated. These other phases recovered fewer compounds from the targeted list, however and were not used.

The sensitivity for the target analytes recovered in Table 3 for tilapia was acceptable. The analyte with the lowest peak area was for AMP which had an area count of 50,000 with a S/N of 340:1, showing that concentration by evaporation of sample extract is not necessary with the RAM approach if 75 µL is injected.

Similar extractions with the RAM procedure were also done for catfish and salmon. Catfish gave very similar results to tilapia but with slightly higher area counts for the β-lactams. The salmon matrix was worse for the dyes BG, MG, CV with even more variable recoveries. The peak width for CTC was also greater in salmon (0.6 min for the quantitation transition). The signal response for the β-lactams were slightly lower in salmon, especially for AMP (around 55% of that for tilapia), although overall recoveries were similar using matrix-extracted standard curves. However, the signal response for the non-polar EMA analyte in salmon was around 5 times higher than that for tilapia. The recoveries for this compound were also less variable and the matrix-matched standard curve had greater linearity (R^2 of 0.96).

The RAM procedure described here was also performed using one additional step of passing the filtered organic extract through a Waters brand OASIS Prime SPE column, which was used in LIB 4516 (8). Since this specialty SPE column (designed to remove phospholipids) requires no preconditioning and is very rapid with only gravity draining needed, its use was investigated to determine if it might improve the procedure. However, it seemed to make no real difference, showing that the RAM procedure achieves similar clean-up. All the analytes listed in Table 3 (except for EMA) met FDA confirmation of identity requirements (12) for product ion ratio abundances using spiked samples compared to matrix-extracted standard curves.

CONCLUSION

Although the RAM procedure described did not recover all the compounds listed in Table 1, the simplicity of the approach was attractive. Future work could investigate using different RAM columns other than the ADS type used here. RAM cartridges are commercially available with bonded ligands possessing hydrophobic and hydrophilic properties (5). There are also RAM sorbents with the trade name of Semi-Permeable Surface® phase (SPS) with different outer particle ligands and various inner surfaces such as nitrile, phenyl and others. There are also newer mixed functional silica materials with both internal and external surfaces consisting of a mixture of hydrophobic and hydrophilic styrene groups. In addition to investigating the performance of other types of RAM columns, other variables could be researched such as using a buffered HPLC mobile phase and optimizing the valve switching times. The RAM approach also might have some usefulness in analyzing veterinary drug residues in milk.

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Table 1. Veterinary drug compounds and their target testing levels for this screening method.

Drug	Class	Target testing level, µg/kg
Doramectin (DOR)	Avermectin	200[a]
Emamectin B1a (EMA)	Avermectin	200 [a]
Ivermectin B1a (IVR)	Avermectin	200 [a]
Amoxicillin (AMOZ)	β-lactam	75
Ampicillin (AMP)	β-lactam	25
Aspoxicillin (ASP)	β-lactam	25
Cloxacillinc (CLOX)	β-lactam	25
Dicloxacillin (DICLOX)	β-lactam	25
Oxacillin (OXAC)	β-lactam	25
Penicillin G (PEN G)	β-lactam	25
Penillic acid	β-lactam	NA - metabolite
Albendazole (ALB)	Benzimidazole	50
Albendazole sulfoxide (ALB SULF)	Benzimidazole	50
Fenbendazole (FEN)	Benzimidazole	50
Fenbendazole sulfone (FEN SULF)	Benzimidazole	50
Cephapirin (CEPH)	Cephalosporin	25
Brilliant Green (BG)	Dye	1
Crystal violet (CV)	Dye	1
Leucocrystal violet (LCV)	Dye metabolite	1
Leucomalachite green (LMG)	Dye metabolite	1
Malachite green (MG)	Dye	1
Ciprofloxacin (CIP)	Fluoroquinolone	5
Danofloxacin (DANO)	Fluoroquinolone	5
Difloxacin (DIFLOX)	Fluoroquinolone	5
Enrofloxacin (ENRO)	Fluoroquinolone	5
Norfloxacin (NOR)	Fluoroquinolone	5
Sarafloxacin (SAR)	Fluoroquinolone	5
Methyl testosterone (M-TET)	Hormone	0.8
Lincomycin (LIN)	Lincomycin	50
Azithromycin (AZI)	Macrolide	50
Erythromycin A (ERY)	Macrolide	50
Erythromycin dehyrated	Macrolide	NA - metabolite
Spiramycin (SPIRO)	Macrolide	50
Tilmicosin (TIL)	Macrolide	50
Tylosin A (TYL)	Macrolide	50
Ketoconazole (KETO)	Nitromidazole	10
Metronidazole (MNZ)	Nitromidazole	10

Florfenicol Amine (FFA)	Phenicol	50[b]
Ormetoprim (ORM)	Potentiator	10
Trimethoprim (TRIMETH)	Potentiator	10
Ethoxyquin (ETHOX)	Preservative	50
Flumequine (FLU)	Quinolone	10
Nalidixic Acid (NAL)	Quinolone	10
Oxolinic Acid (OXO)	Quinolone	10
Sulfacetamide (SAA)	Sulfonamide	10
Sulfachloropyridazine (SCP)	Sulfonamide	10
Sulfaclozine (SULC)	Sulfonamide	10
Sulfadiazine (SDZ)	Sulfonamide	10
Sulfadimethoxine (SDM)	Sulfonamide	10
Sulfadoxine (SDX)	Sulfonamide	10
Sulfaethoxypyridazine (SEP)	Sulfonamide	10
Sulfamerazine (SMR)	Sulfonamide	10
Sulfamethazine (SMZ)	Sulfonamide	10
Sulfamethoxazole (SMOZ)	Sulfonamide	10
Sulfamethoxypyridazine (SMP)	Sulfonamide	10
Sulfamonomethoxine (SULFMON)	Sulfonamide	10
Sulfapyridine (SPD)	Sulfonamide	10
Sulfaquinoxaline (SQX)	Sulfonamide	10
Sulfathiazole (STZ)	Sulfonamide	10
Chlortetracycline (CTC)	Tetracycline	75[c]
Doxycycline (DC)	Tetracycline	75 [c]
Oxytetracycline (OTC)	Tetracycline	75 [c]
Tetracycline (TC)	Tetracycline	75 [c]

[a] Current FDA program recommends TTL of 10 µg/kg (5)
[b] FDA tolerance of 1 mg/kg for FFA as marker residue in aquaculture species
[c] FDA tolerance of 2 mg/kg for sum of OTC, CTC, and TC in finfish and lobster

Table 2. Pump flow rates

RAM Column Pump

Step	Total Time (min)	Flow rate (µL/min)	%A	%B
0	0	750	98	2
1	8*	750	98	2
2	17*	750	70	30
3	20	750	5	95
4	22	750	98	2
5	23	750	98	2

HPLC Column Pump

Step	Total Time (min)	Flow rate (µL/min)	%A	%B
0	0	1250	95	5
1	8*	1250	95	5
2	12	1250	70	30
3	17*	1250	70	30
4	20	1250	2	98
5	22	1250	95	5
6	23	1250	95	5

* Switching Valve (SV) times: 0-8 min load RAM column, 8-17 min backflush RAM onto HPLC column-see Figure 2. A=0.1% formic acid in water, B=acetonitrile.

Table 3. Performance summary of target veterinary drug compounds using the RAM procedure on Tilapia at 1X-3X levels.

Compound	**Retention Time (min)[a]**	**Recovery Range (%)**	**Avg. R^2 of Std. Curve**	**MS^2 Quantitation transition Used (m/z)**
ALB SULF	13.2	62-109	.990	282→240
AMP	12.0	90-110	.995	350→106
BG	20.1	70-250	.800	385→341
CTC	13.6	92-110	.995	479→444
CIP	12.5	92-108	.995	332→288
CLOX	20.0	66-122	.995	436→277
CV	19.9	53-112	.950	372→356
DANO	12.6	91-107	.995	358→283
DICLOX	20.3	85-150	.995	470→160
DIFLOX	13.2	89-106	.998	400→356
DOXY	13.8	96-107	.999	445→154
EMA	20.1	86-200	.890	886→302
ENRO	12.7	88-97	.997	360→316
ERYTH	15.4	93-114	.995	734→158
FEN SULF	16.5	69-111	.998	332→300
FLU	19.0	75-111	.997	262→244
LIN	11.8	92-107	.997	407→126
MG	19.6	55-108	.970	329→313
NAL	18.1	90-108	.999	233→187
NOR	12.3	97-103	.997	320→276
OXAC	19.7	68-108	.910	402→160
OXO	15.0	85-112	.999	262→244
OTC	12.5	95-105	.999	461→426
PENILLIC[b]	12.7	92-118	.998	335→176
PEN	17.4	91-120	.995	335→160
SARA	13.0	93-106	.998	386→342
SCP	15.6	93-108	.999	285→156
SDM	15.8	90-111	.998	311→156
SDX	14.4	85-107	.998	311→156
SEP	14.4	94-105	.998	295→156
SMX	14.4	99-109	.998	254→156
SMP	13.8	103-111	.999	281→156
SQX	15.8	85-107	.999	301→156
SPIRO	13.1	88-112	.999	422→174
TET	12.8	95-103	.999	445→410
TIL	13.8	87-111	.999	869→174
TRIM	12.3	85-101	.996	291→230
TYL	16.7	95-107	.999	916→174

[a]The retention times listed here include the 8-minute RAM column loading phase time.
[b]Penillic acid is a degredation product of Penicillin G.

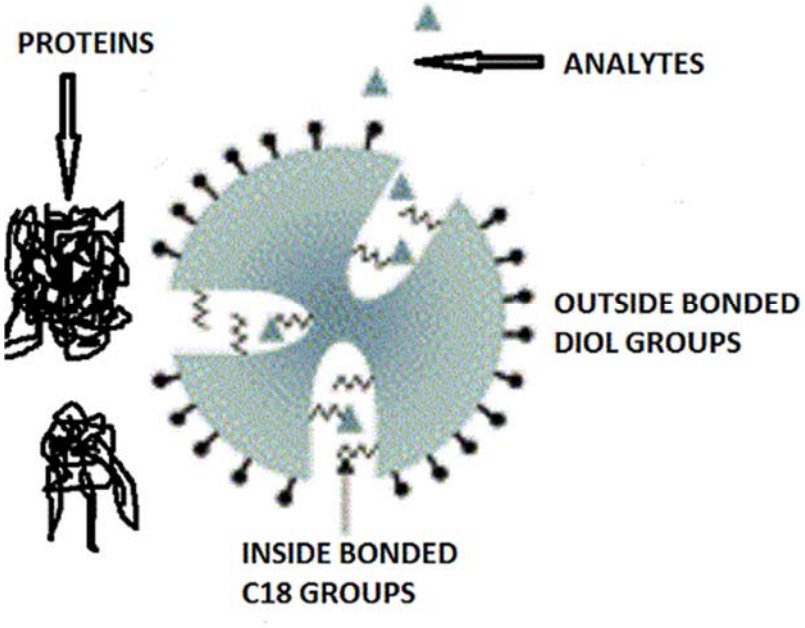
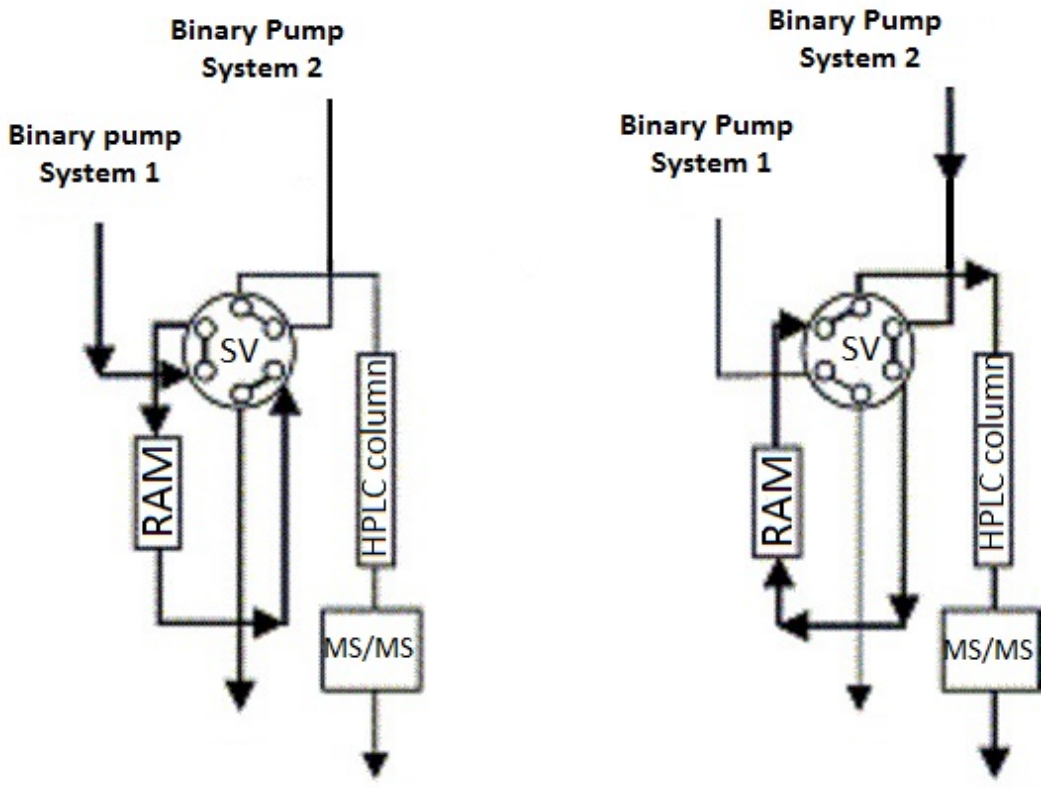


Figure 1. Chromatographic processes occurring at one 25 µm RAM particle with a 60 Å pore size. Figure adapted from (4).



"Load Position" "Backflush Position"

Figure 2. Dual pump system flow path using an on-line RAM column and analytical HPLC column. (Flow path on left loads and concentrates the analytes onto the head of the RAM column. Flow path on right back flushes the analytes over to the HPLC analytical column). SV=switching valve.