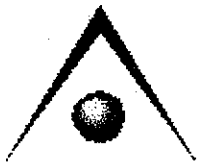

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Fast Gradient Elution Reversed-Phase HPLC with Diode-Array Detection as a High Throughput Screening Method for Drugs of Abuse

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Abstract

A new approach has been developed by modifying a conventional gradient elution liquid chromatograph for the high throughput screening of biological samples to detect the presence of regulated intoxicants. The goal of this work was to improve the speed of a gradient elution screening method over current approaches by optimizing the operational parameters of both the column and the instrument without compromising the reproducibility of the retention times, which are the basis for the identification. Most importantly, the novel instrument configuration substantially reduces the time needed to re-equilibrate the column between gradient runs, thereby reducing the total time for each analysis. The total analysis time for each gradient elution run is only 2.8 minutes, including 0.3 minutes for column re-equilibration between analyses. Retention times standard calibration solutes are reproducible to better than 0.002 minutes in consecutive runs. A corrected retention index was adopted to account for day-to-day and column-to-column variations in retention time. The discriminating power and mean list length were calculated for a library of 47 intoxicants and compared with previous work from other laboratories to evaluate fast gradient elution HPLC as a screening tool.

Introduction

Drug Screening Technologies

Delivering competent analytical judgment on samples in a timely manner is becoming more difficult as the sample load in forensic laboratories continues to increase, despite the fact that economic pressures do not permit a concomitant increase in staff size. These pressures are prompting the development of new analytical technologies, which can deliver high quality qualitative and quantitative information in a high throughput environment. Historically, gradient elution liquid chromatography with diode array detection (LC-DAD) has been a common method for screening and identification of drugs of abuse in biological samples because the technique is relatively inexpensive, has tremendous chemical selectivity, and can produce precise retention time data under well controlled conditions [Herzler, 2003]. Indeed, considerable effort has been dedicated over the past two decades to improving the specifications of discriminating power (DP) and mean list length (MLL) of the technique. Hertzler and co-workers have used two different isocratic LC methods with DAD detection that provides two relative retention times, along with a database of 2,682 toxicologically relevant compounds to achieve a DP of 0.9999 and a MLL of 1.253[Herzler, 2003]. The capability of the LC-DAD systems discussed by Herzler is most impressive; however, this predictive power does come at the cost of substantial analysis time; specifically, their isocratic analyses were 30 minutes long per isocratic analysis. Gas chromatography with a flame ionization detector (GC-FID) or a nitrogen-phosphorus detector (GC-NPD), and

enzyme immunoassay (EIA) have been used most frequently for screening samples for drugs of abuse. EIA has less specificity than chromatographic techniques and is prone to cross-reactivity; GC screening methods have long run times (30 to 40 minutes or more) and involve lengthy sample preparation steps including derivatization in some cases [Ferrara, 1994, Foerster, 1978, Kroener, 2003].

The hyphenated technique of GC coupled with mass spectrometry (GC-MS), and more recently LC coupled with spectrometry (LC-MS), have been used as screening methods as well, as mass spectrometry gives high selectivity and sensitivity compared UV absorbance detectors. However, the high cost of instrumentation and the requisite operator expertise limit wide adoption as screening methods. For GC-MS analysis, derivatization of the sample is often required, and in LC-MS optimal ionization conditions are analyte dependent and can be significantly different(references). Because of these limitations of GC-MS and LC-MS approaches, a rapid screening methods based on LC-DAD can be quite valuable.

Given the unparalleled selectivity of the MS detector, GC-MS and LC-MS have been widely used as the method of choice for "confirmatory analyses" in the forensic laboratory [Bogusz, 1999; Eivier, 2003]. In their Forensic Toxicology Laboratory Guidelines, the American Academy of Forensic Sciences explicitly states that MS is recommended, whenever practical, as the confirmatory technique [SOFT/AAFS, 2002]. Given that chromatography coupled with mass spectrometry will undoubtedly be the confirmatory

technique of choice, a great need exists for a screening technique for preliminary analysis that has the discriminating power of the LC-DAD approach discussed above, but which maintains the speed of the immunoassay-based technique to enable the analysis of several hundreds of samples per day. We believe the order-of-magnitude improvement in the speed of gradient LC-DAD achieved here meets these criteria.

Fast Gradient Elution LC

Historically the major limiting factor in terms of speed in gradient elution LC is the time needed to re-equilibrate the column. To achieve high throughput, reduction of the total analysis time is necessary; the total analysis time is the sum of the gradient program time and the between run column re-equilibration time. Schellinger et al. [Schellinger, 2005] have determined how to minimize re-equilibration time without compromising the extraordinary high precision of retention times ($\pm$ 0.002min) that they were able to achieve under carefully controlled conditions. In the present study, the instrument was modified to significantly reduce the flush-out volume of the system; this greatly reduces the apparent column re-equilibration time, thereby ultimately reducing the total analysis time for each complete gradient elution cycle. We have developed fast gradient elution HPLC with a total analysis time of less than 4 minutes capable of providing relative precision in retention time comparable to the much longer (20-30 minutes) LC analyses currently in use as a screening tool.

Evaluation of Discriminating Power

The major concern with analytical technologies being used as screening methods is their ability to discriminate between target analytes based solely on the measured gradient retention time. For the evaluation of discriminating power, numerous retention indices have been developed that attempt to correct for long-term, systematic variations in analyte retention times [Smith, 1987,2]. The 1-ntiroalkane scale has been most commonly used [Bogusz, 1991(2), 1994, 1995, 3-6]; however, Bogusz and coworkers have shown that the use of basic drugs as index markers can significantly improve the precision of relative retention indices, particularly when comparing data from different columns or instruments [Bogusz, 1993,7]. The list length (LL), introduced by Schepers and coworkers [Schepers, 1983, 8], is defined as the number of compounds (n) from the set of all target analytes (q) that are indistinguishable from a given target analyte under the conditions of the screening method; the mean list length (MLL) is simply the average value of the list length across all of the compounds of interest.

$$MLL = \frac{\sum_{i}^{q} n_i}{q} \qquad (1)$$

The discriminating power (DP), introduced by Moffat et al.[Moffat.1974, 9], is the probability that two analytes selected randomly from the complete set of target analytes will be indistinguishable under the conditions of the screening method, where p is the total number of indistinguishable pairs of compounds:

$$DP = 1 - \frac{2p}{q(q-1)} \quad (5)$$

The DP and MLL are fundamentally different, but both are informative and common figures of merit which are functions of both the selectivity of the separation and the precision of the retention time measurement. Therefore, both DP and MLL were calculated for the 47 intoxicants studied in this work to determine the effectiveness of our fast gradient LC system as a screening tool.

Experimental

Instrumentation

The following system was assembled to allow fast, reproducible gradient elution with narrow-bore columns; it will be referred to as system A. Explicit details regarding the operation of a system of this type were discussed previously [Schellinger, 2005]; what follows is a description of the *essential* components of the system. To reduce the required re-equilibration time when using narrow-bore columns, a very low dwell volume HPLC system was assembled using two complete binary HP1090 (DR5) pumping systems (Agilent Technologies, Palo Alto, CA) (pumps A and B in Figure 1), two pneumatically actuated six-port Rheodyne valves (valves A and B in Figure 1) (Model 7000, Rohnert Park, CA), and a HP1040 stand-alone diode-array detector. The function of valve A is simply to allow delivery of the desired solvent composition from either of the binary pumps A or B as dictated by the operator. The function of valve B is to deliver the sample to be analyzed to

the column head at the time of injection. Gradient elution for subsequent injections was performed by alternately selecting either pump A or B (using valve A) as the pump delivering the solvent gradient. The first solvent gradient is delivered by pump A, followed by actuation of valve A to allow delivery of solvent from pump B to the column head. This arrangement allows immediate re-equilibration of the column with the initial eluent without waiting for strong solvent from the first gradient to be flushed from the system. This greatly reduces the dwell volume of the system. Initial solvent is delivered from pump B through the column for a specified re-equilibration time, which is then followed by injection of the second sample and delivery of the second solvent gradient to the column using pump B. This process of delivering solvent gradients from alternating pumps can then be repeated as many times as necessary to complete the analysis of a large number of samples. The temperature of the eluent entering the HPLC column was controlled to 40 ± 0.1 °C using a prototype eluent pre-heater and column heating jacket that were the generous gifts of Systec Inc. (New Brighton, MN). A commercially available version of this column heating apparatus is available from Metalox, Inc. (Anoka, MN). All timed events in this system (i.e., start of each binary pumping system, actuation of valves A and B, and initiation of data collection) were controlled with LabView version 6.0 and a 6024E data acquisition card from National Instruments (Austin, TX). We estimate the dwell volume of this system to be in the range of 10-20 μL, as only the tubing connecting valves A and B contribute to this volume.

A re-equilibration time of 0.30 minutes was used between gradient analyses, which corresponds to approximately two column volumes of initial eluent for a 50 mm x 2.1 mm i.d. column at a flow rate of 1.0 mL/min. In this way a total analysis time of 2.8 minutes per gradient analysis was achieved.

While system A described above was used for much of the work described here, some development work was done using the same system with only one binary pumping system; this system will be referred to as system B. In this case the dwell volume is 0.35 mL (at 100 bar) as measured by a previously established method [Snyder, Glajch]. To allow comparison of data acquired under these conditions to data acquired using system A, the effective dwell volume of system B was decreased to zero by delaying injections relative to the start of the gradient program in the pumping system (18 seconds at a flow rate of 1.0 mL/min.). Because of the much larger actual dwell volume of system B compared to system A, a re-equilibration time of 1.5 minutes was used to allow the final eluent to be flushed out of the pumping system before beginning subsequent gradient analyses. With system B a total analysis time of 4.0 minutes was required per gradient analysis.

Reagents

The uracil and nitroalkane test solutes were of reagent grade or better and were used as obtained from the manufacturer without further purification. Acetonitrile was obtained from Burdick and Jackson (Muskegon, MI) or Sigma-Aldrich (St. Louis, MO). Sodium

dihydrogen phosphate was from JT Baker (Philipsburg, NJ), and sodium perchlorate was obtained from Sigma-Aldrich; sodium monohydrogen phosphate and perchloric acid (70%) were obtained from Fisher Scientific (Fairlawn, NJ). HPLC grade water was obtained in-house from a Barnstead Nanopure Deionizing system (Dubuque, IA). This water was boiled to remove carbon dioxide and cooled to room temperature before use.

All eluents were prepared gravimetrically (± 0.01 g); with the exception of the 20 mM perchloric acid in water; all eluents were passed through a 0.45 μm nylon membrane filtration apparatus (Lida Manufacturing Inc.; Kenosha, WI) immediately before use. All eluents were passed through a vacuum degasser (P/N 0001-6501) which was a generous gift of Rheodyne Inc. (Rohnert Park, CA) immediately prior to entering the pumping system of the HPLC system.

Columns

The columns used in this work were 50 mm x 2.1 mm i.d., and were prepared in-house using bulk packing material graciously provided by the manufacturer. The packing materials were slurried in tetrahydrofuran and sonicated (model PC3, L&R Manufacturing, Kearny, NJ) for 20 minutes prior to packing. All columns were packed using the downward slurry technique using tetrahydrofuran as the driving solvent. The pressure applied during packing was slowly increased from 500 – 7,000 psi during the first two minutes of packing, and maintained at 7,000 psi until 70 mL of the driving solvent had been collected. The

stainless steel column hardware was obtained from Isolation Technologies (Hopedale, MA). The column packing pump was a Haskel 16501 high-pressure pump (Haskel International Inc., Costa Mesa, CA). Stable Bond-C18 packing material (referred to as SB-C18, 5 μm, Lot # B04082) was a generous gift of Agilent Technologies (Palo Alto, CA).

Drug Standards

Standards of all of the drugs studied in this work were purchased from Cerilliant (Round Rock, TX) as either 100 μg/mL or 1 mg/mL solutions of drug in methanol, with the exception of amitriptyline which was purchased from Sigma-Aldrich as a solid and used without further purification. Analytical samples of individual drugs were prepared by diluting to ca. 20 μg/mL in the initial eluent, which was 10/90 (v/v) acetonitrile/buffer. Analytical samples of drug mixtures were prepared by first combining appropriate volumes of each drug standard in methanol, evaporating the methanol under a gentle stream of nitrogen, and finally reconstituting the sample in the initial eluent.

Chromatographic Conditions

The bulk of the work was carried out with the SB-C18 column and the following gradient program. An A solvent containing 20 mM perchloric acid in water and B solvent containing 20 mM perchloric acid in 80/20 (v/v) acetonitrile/water were used to produce a gradient profile from 12.5-56.3-12.5 % B from 0-2.50-2.51 minutes, at a flow rate of 1.0

mL/min. unless otherwise noted. The gradient re-equilibration time varied depending on which instrument configuration was used, as discussed above. Injections were made from a pneumatically actuated (with helium) six-port Rheodyne valve and a 10 μL sample loop.

Data Acquisition and Analysis

Chromatographic signals and associated UV spectra (200-400 nm) were acquired and analyzed using Chemstation software (rev. A.10.01, Agilent Technologies, Palo Alto, CA). Retention times reported by the Chemstation were exported to Microsoft Excel (Microsoft Corporation, Redmond, WA) for further analysis. The excellent precision of retention time in some cases produced a standard deviation of less than 0.001 minutes. A macro was obtained from the User Contributed Library of Agilent Technologies that allowed the exporting of retention times with up to ten decimal places, which enabled the subsequent calculation of standard deviations of retention time to more than three decimal places.

Result and Discussion

Development of fast gradient elution HPLC

The ultimate goal of this study was to develop fast gradient HPLC-DAD as a screening tool for drugs of abuse, with total analysis times of less than 4 minutes, and capable of providing high information content in terms of peak capacity and retention time precision. In the development of this type of methodology, selection of a stationary phase is paramount. In order to determine the best-suited stationary phase for this work, we evaluated four

different commercially available phases. Several characteristics of an ideal stationary phase and a comparison of columns tested are shown in Table 1. First, the ideal stationary phase would be highly retentive because many of the drugs are very hydrophilic and thus may not be retained on a weakly retentive column. Secondly, as the aqueous eluent contains acid to improve the peak shape of basic drugs, it also requires the column to be acid-stable to prevent long term retention drift due to stationary phase loss. The last requirement is high sample loadability. The higher the loading capacity of the phase, the less effect the sample mass will have on the apparent retention time. Compared to the other phases examined in this work (data not shown) the SB-C18 provides the best compromise of attributes, and thus it was chosen for this study.

Upon selection of the specific stationary phase, the gradient HPLC conditions were chosen to maximize the chromatographic selectivity for selected opiate and amphetamine derivatives, within the shortest total analysis time. Acid was added to the aqueous eluent to improve the peak shape of the basic analytes. Perchloric acid was chosen because it also increases the retention time of some of the weakly retained drugs by forming ion pairs; additionally, perchlorate has a very low UV background signal, which provides a clean baseline to allow for improved limits of detection (LOD). It is clear from several studies of the effect of operational parameters on peak capacity in gradient elution RPLC that there is a compromise between peak capacity and analysis time. In choosing the gradient profile, we

first considered the trade-off between the speed of analysis and the DP. It is obvious that a longer gradient elution can produce a higher DP due to higher peak capacity, however the time for analysis becomes longer. As a result of the compromise between speed and identification power, a gradient profile of 10-45% (v/v) acetonitrile-water was chosen.

The cycle time for a gradient elution analysis using a conventional HPLC system is typically 30 minutes or longer; this results in unacceptably low throughput. We reduced the total analysis time by modifying the instrument as described in the experimental section. For one binary pump HP1090 system (referred to as system B in experimental section), 1.5 minutes of re-equilibration time is required before starting the subsequent gradient elution, therefore the total analysis time is four minutes. We deemed this acceptable, however as the cycle time is reduced, the fraction of the cycle time used by the system flush-out and column re-equilibration becomes greater. Thus, we attempted to further decrease the flush-out volume by configuring the system B with an additional binary pump and 6-port 2-position valve in order to improve the speed of the analysis. This system is shown schematically in Figure 1. As a result, the flush-out volume of the system is reduced by 85% compared to the system B. Because of this significantly reduced flush-out volume, the time required to re-equilibrate the column with the initial eluent is decreased to 0.3 minute. Consequently under the conditions of this experiment, the total analysis time is reduced by approximately 30%. Ultimately we can perform the gradient analysis in less than 3 minutes, which can

significantly increase throughput.

A major concern when using the two-binary pump system is the similarity of the gradient profiles generated by two different pumps. Considerable differences were seen in the retention times produced by the two different pumps. Therefore, we sought for a way to correct the retention times based on a retention index scheme, as discussed below.

Application of Retention Index

We developed a set of neutral and "standard" drug compounds that covered the range in retention time of all 47 intoxicants (see Table 2) to be used as a control to check the system in order to observe within-day, day-to-day, and column–to-column variations. We refer to the neutral compounds as primary standards, the drug compounds as secondary standards, and the entire group as calibration standards. Since we desired to at least maintain the DP of the fast gradient method compared to other "slow" HPLC methods, the precision of the retention time is important as it ultimately will have a large impact on the discriminating power. The within-day standard deviations of the retention times of the calibration standards are shown in Figure 2. These standard deviations are based on four consecutive replicate injections. Despite the short column re-equilibration time of 0.3 minutes, the overall precision of retention time is excellent; all standard deviations are less than 0.002 minutes.

In addition to the random within-day variation, there are several other factors that can contribute to the variability of retention time over longer time scales (i.e., months or years),

which will ultimately limit the predictive capability of the method as discussed above. We have assessed day-to-day and column-to-column precision of retention time as being representative of the medium and long-term variability of retention time of the target analytes. These results are shown in Table 3 along with the within-day precision values for comparison. It is clear from these data that the width of the confidence interval for the retention values of these compounds is dominated by the day-to-day and column-to-column changes in retention. Since the list length depends on the total variance of the retention measure that is used for identification of unknowns, it is desireable to minimize this variance as much as is reasonably possible. As shown in Table 3, the within-day variability is negligible, however the day-to-day and column-to-column variability must be dealt with.

We adopted a retention index approach as a method of correction for changes in retention time of the intoxicant compounds studied in this work. The general approach has been described in detail previously by Smith. While the general approach is dependent on the use of a set of primary standards (see Table 2A), Bogusz and coworkers have described a specific application of the retention index that is dependent on the use of a set of secondary standards that emulate the characteristics of the target analytes; this approach has been shown to reduce long-term variance in the apparent retention measure of target analytes.

First, the retention time of each target analyte in Table 2B was converted to an 'observed retention index' value (RI_{obs}) using equation 3[Smith]:

$$RI_{obs,i} = 100n + 100\left(\frac{t_{r,i} - t_{r,n}}{t_{r,n+1} - t_{r,n}}\right) \quad (3)$$

where n is the number of carbons in the primary standard compound eluting prior to the target analyte, $t_{r,n}$ and $t_{r,n+1}$ are the retention times of the primary standards eluting immediately before and after the target analyte, and $t_{r,i}$ is the retention time of the target analyte itself. Based on a comparison of the relative standard deviations of the retention times and observed retention indices in Table 4, the day-to-day and column-to-column reproducibility of retention is considerably improved by using the retention index approach. There is a roughly six-fold improvement in the day-to-day and column-to-column standard deviations, however the resulting standard deviations are still roughly two- to five-fold larger than the within-day precisions of retention index.

We attempted further corrections to the RI values by linear interpolation of the retention index using the secondary drug standards in Table 2b. Following the method of Bogusz et al.[Bogusz, Galanos], "corrected" RI values are calculated using the following equations:

$$a = \frac{RI^{o}_{s+1} - RI^{o}_{s}}{RI_{obs,s+1} - RI_{obs,s}} \quad (4)$$

$$b = aRI^{o}_{s+1} - RI_{obs,s+1} \quad (5)$$

$$RI_{i,corr} = aRI_{i,obs} + b \quad (6)$$

where RI^{o}_{s} and RI^{o}_{s+1} are the retention indices of the secondary standards eluting immediately

prior to and following analyte *i*, and $RI_{obs,s}$ and $RI_{obs,s+1}$ are the retention indices of the secondary standards eluting immediately prior to and following compound *i*, calculated for the day of analysis using equation 3. The improvement in the standard deviation of the retention index is best demonstrated by comparison of the average standard deviation of retention index for the column-to-column data, before and after correction. Since data for morphine were not collected as part of this dataset, and calculation of a corrected retention index is not possible for the least and most retained secondary standards, an average standard deviation was calculated for the secondary standards including benzoylecognine, zolpidem, diazepam, clonazepam, and temazepam. The average column-to-column standard deviation of retention index was 0.34%, while the same average for the corrected retention index values was 0.06%. The value of 0.06% is on the order of the relative within-day standard deviation of retention index; we certainly cannot expect better performance than this if we assume that the within day precision is controlled by uncontrollable, random variations in retention time.

The RI was also used as a means of correcting for the observed differences in retention times of the target analytes obtained from separations where consecutive solvent gradient profiles were generated using two different binary pumping systems in order to implement the fast gradient elution HPLC system described in the experimental section. The percent differences in retention times and retention indices for 47 intoxicants is plotted as a function of retention time in Figures 3 and 4 respectively. The comparison of the two plots

shows that the variability in the data is reduced considerably by using the RI approach. The average %RI difference is just 0.05, which is roughly only $1/30^{th}$ of the average variability in the long term changes in retention time (see Table 4). However, due to the negative minimum value of the % relative RI difference, it is more rational to use the median rather than the mean for comparison. The median value of the % relative RI difference is 0.16, which is still very small compared to the median relative retention time difference of 1.2%. It is reasonable to expect that further correction of retention indices of the target analytes by linear interpolation based on secondary calibration standards would further reduce the observed differences between data obtained using the two pumping systems to the level of the within-day precision of the retention index.

Evaluation of identification power

To validate the potentiality of this approach as a screening method, we assessed the discriminating power by calculating the DP[Moffat 1974] and MLL[Schepers 1983] values for the current work. These figures of merit show that our approach is not only much faster compared to the conventional HPLC approach, but is also a precise and powerful method for identification of intoxicants. In previous work involving 56 acidic drugs, Bogusz et al. achieved a DP of 0.747 and a MLL of 8.38 of based solely on RI, assuming that the standard deviation of the RI for HPLC was fixed at 10 units (on a scale of roughly 600) [Maier, 1995].

These results were obtained by using a conventional HPLC system and a gradient time of 35 minutes plus additional time for re-equilibration.

We calculated the MLL and DP for our approach as follows. To estimate the size of the error window we averaged the standard deviations of the retention indices obtained from the day-to-day and column-to-column variability studies, and excluded the within-day variation since it was negligible in comparison. Based on our estimates of the error window the calculated MLL and DP values are 1.19 and 0.974, respectively. The values are remarkably improved compared to those obtained by Bogusz et al. This comparison clearly shows that our new approach is a significant improvement in gradient LC as a screening tool in terms of both analysis speed and identification power.

Conclusions

The modified gradient elution HPLC system gives excellent within-day retention time precision (±0.002 min) despite the very short overall analysis time (less than four minutes). Although there is greater variability of retention time observed from day-to-day and from column-to-column, this variability can be mitigated by using a retention index scheme. Using retention index rather than retention time as the characteristic measure of when analytes elute from the HPLC column, the variability in this measure is reduced to the level of the run-to-run reproducibility if the retention index values are corrected using a simple

linear interpolation along with secondary standards that resemble the analytes. Very fast gradient elution analyses can be achieved using a modified system employing two independent binary pumping systems. The physical attributes of each of these pumping systems are apparently different enough to cause significant differences in gradient elution retention time. However, when a retention index scheme corrected by linear interpolation and secondary standards is used these differences are generally minimized to near the level of within day retention index precision.

The effectiveness of our system as a screening technique was shown by comparing the DP and MLL of our approach to those from the literature. Our approach not only greatly decreased the total analysis time, but also proved to have better power for the identification of target compounds. The improvement in speed and discriminating power of the technique would allow the analysis of several hundreds of samples per day.

Table 1. Characteristics of the ideal stationary phase for this application and that of commercially available phases

Column	Retention	Efficiency	Stability	Loadability
SB-C_{18}	+	++	+	+
Carbon –ZrO_2	++	+	++	-
Hamliton PRP-1	+	-	++	?
Xterra MS-C_8	+	++	-	+

++: very good +: good -: fair

Table 2. Neutral compounds and drugs used in this study along with index number

a. Neutral compounds

Index	Compound Name
A	Uracil
B	Nitromethane
C	Nitroethane
D	Nitropropane
E	Ethyphenone
F	Nitrobutane
G	Propylphenone
H	Nitropentane
I	Butylphenone
J	Nitrohexane

b. Drugs

Index	Compound Name	Index	Compound Name
1	Morphine*	27	7-Aminoclonazepam
2	Oxycodone*	28	Methcathinone
3	Benzoylecgonine*	29	PMA
4	Zolpidem*	30	Nitrazepam
5	Diazepam*	31	Triazolam
6	Clonazepam*	32	Cathinone
7	Temazepam*	33	7-aminoflunitrazepam
8	Amitriptyline*	34	Hydrocodone
9	Pseudoephedrine	35	Desalkylflurazepam
10	Methamphetamine	36	Alprazolam
11	MDEA	37	Cyclobenzaprine HCl
12	Chlordiazepoxide	38	Amphetamine
13	2-hydroxyethyflurazepam	39	MDMA
14	Flurazepam	40	PMMA
15	Lometazepam	41	Nordiazepam
16	Hydromorphone	42	Oxazepam
17	Codeine	43	Midazolam
18	MDBD	44	Oxymorphone
19	Lorazepam	45	Ephedrine
20	Prazepam	46	6-Acetylmorphine
21	Phenylpropanolamine	47	Bromazepam
22	MDA	48	Estazolam
23	Phentemine	49	Hydroxyalprazolam
24	Clobazam	50	Hydroxymidazolam
25	Flunitrazepam	51	Hydroxytriazolam
26	Sertraline	52	Halazepam

* Drugs used as secondary standards

Table 3. Comparison of within-day, day-to-day, and column-to-column standard deviations of retention time (min.) of primary and secondary calibration standards

Compound Name	Day-to-day (n*=10)	Column-to-column (n=5)	Run-to-run (n=4)	Compound Name	Day-to-day (n=10)	Column-to-column (n=5)	Run-to-run (n=4)
Uracil	0.0008	0.0042	0.0008	Morphine	0.0036	-	0.0007
Nitromethane	0.0006	0.0047	0.0008	Oxycodone	0.0076	0.0169	0.0003
Nitroethane	0.0019	0.0067	0.0009	Benzoylecgonine	0.0100	0.0247	0.0007
Nitropropane	0.0062	0.0155	0.0010	Zolpidem	0.0108	0.0308	0.0007
Ethyphenone	0.0100	0.0267	0.0010	Diazepam	0.0118	0.0291	0.0011
Nitrobutane	0.0120	0.0305	0.0013	Clanazepam	0.0113	0.0302	0.0009
Propylphenone	0.0123	0.0337	0.0012	Temazepam	0.0105	0.0300	0.0010
Nitropentane	0.0147	0.0356	0.0013	Amitriptyline	0.0126	0.0285	0.0010
Butylphenone	0.0125	0.0334	0.0009				
Nitrohexane	0.0148	0.0311	0.0014				

* Number of replicates

a. Column: 50 mm x 2.1 mm i.d. Stable bond C18; Flow rate 1 mL/min.; Gradient elution from 10-45% B in 4 minutes, where A is 20 mM perchloric acid in water, and B is 20 mM perchloric acid in 80/20 (v/v) acetonitrile/water; Temperature, 40°C, Injection volume, 10µl; Detection UV absorbance at 210 nm.

Table 4. Comparsion of percent relative within-day, day-to-day, and column-to-column standard deviations of retention time and observed retention index for secondary calibration standards

	Within-Day		Day-to-Day		Column-to-Column	
Compound Name	t_r	Ri_{obs}	t_r	Ri_{obs}	t_r	Ri_{obs}
Morphine	0.26	0.35	1.38	1.90	N/A	N/A
Oxycodone	0.05	0.03	1.30	0.33	2.83	0.79
Benzoylecgonine	0.08	0.03	1.13	0.11	2.73	0.46
Zolpidem	0.05	0.02	0.82	0.10	2.30	0.41
Diazepam	0.07	0.04	0.79	0.18	1.93	0.33
Clonazepam	0.05	0.03	0.65	0.12	1.72	0.25
Temazepam	0.05	0.03	0.53	0.16	1.51	0.25
Amitriptyline	0.04	0.03	0.56	0.11	1.25	0.28

a. Chromatographic conditions and number of replicates were the same as those given in Table 3.

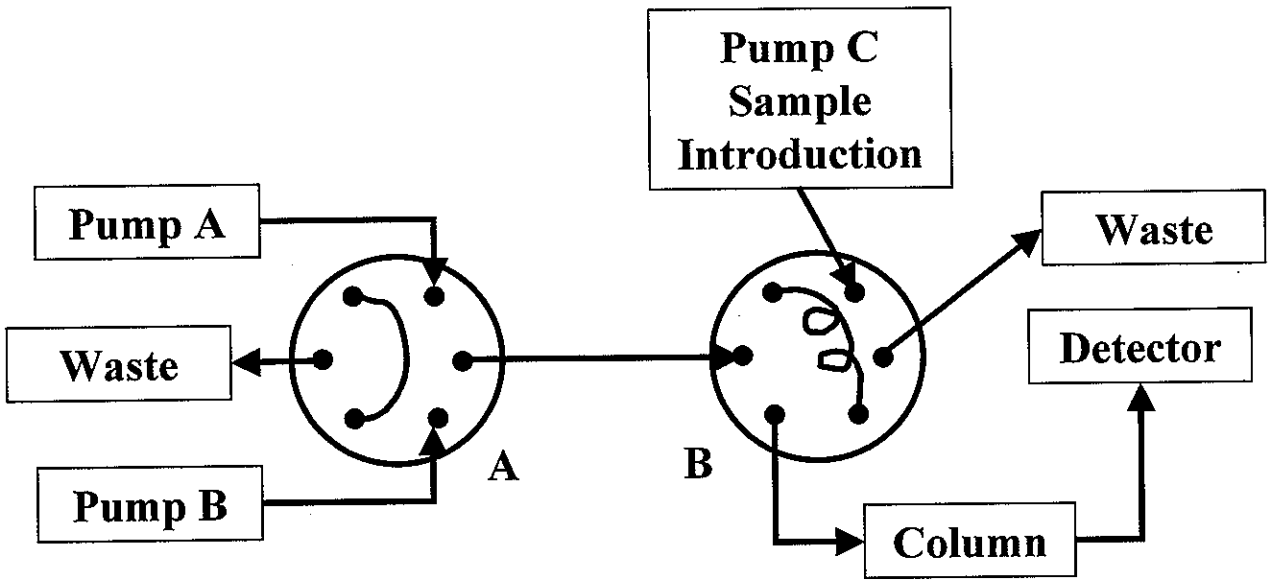

Figure 1. Schematic representation of a modified HPLC system that allows fast column re-equilibration between gradient analyses

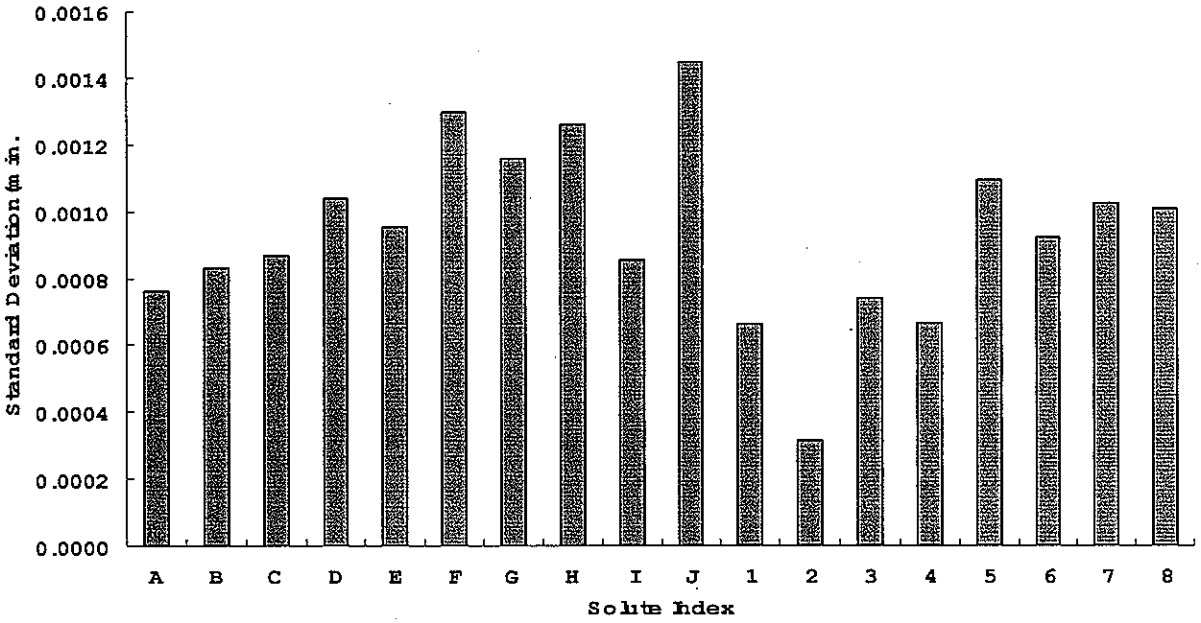

Figure 2. Plot of run-to-run precision of neutral and standard drug compounds. The solute index follows as labeled in Table 2, and the conditions were the same as in Table 3.

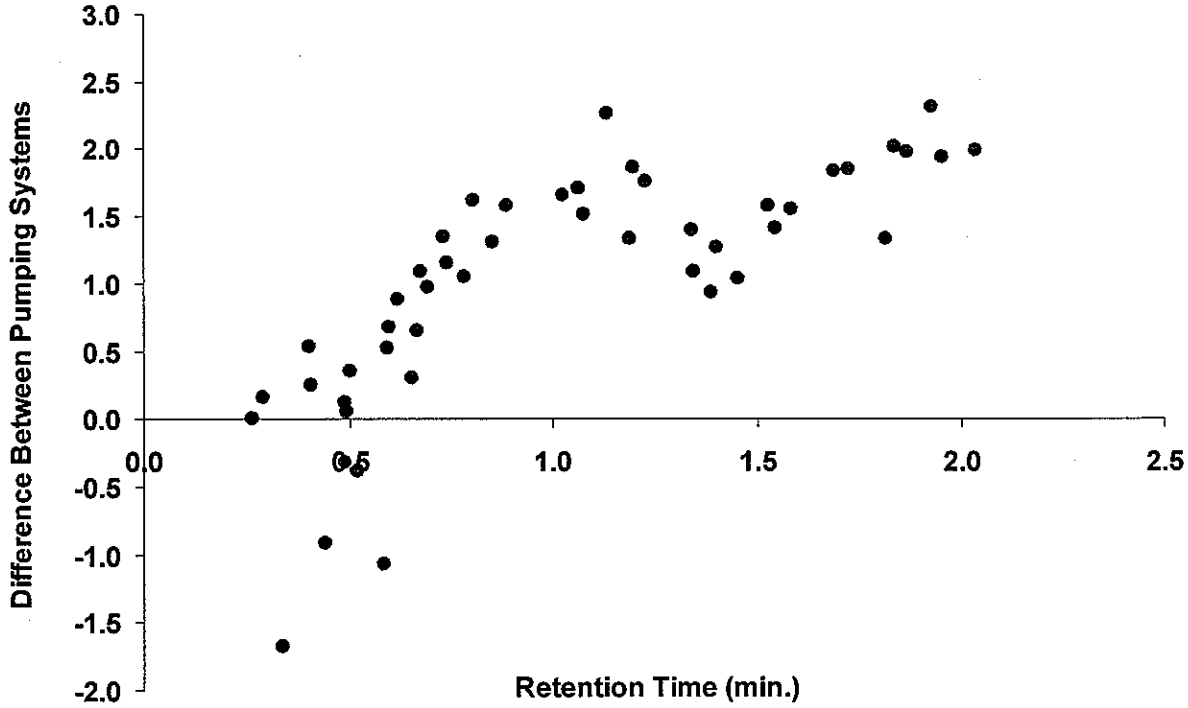

Figure 3. Plot of percent difference in retention time of drugs 1-48 (Table 2B) on pumping system A vs. pumping system B. The conditions were the same as in Table 3 except that the gradient time is 2.8 minutes.

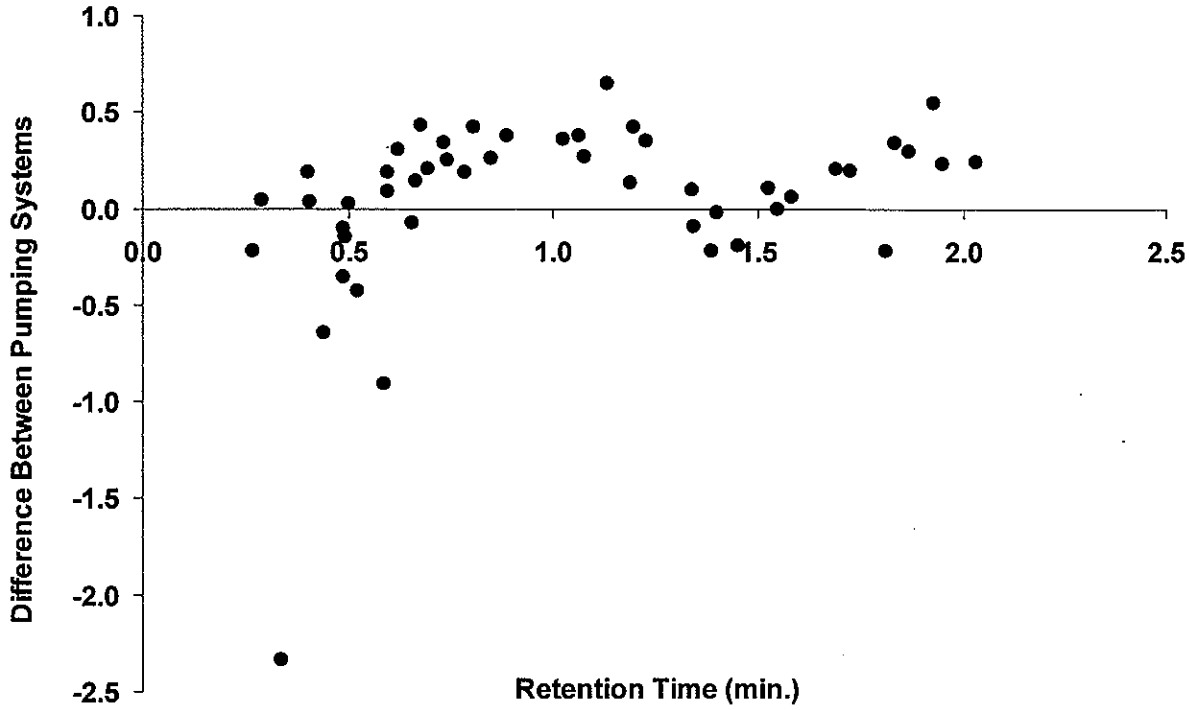

Figure 4. Plot of percent difference in retention index of drugs 1-48 (Table 2B) on pumping system A vs. pumping system B. The conditions were the same as in Table 3 except that the gradient time is 2.8 minutes.