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A test to determine the nature and presence of the memory effect columns packed with the amylose tris(3,5-dimethylphenylcarbamate) stationary phase

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ABSTRACT

Acid/base mobile phase modifiers affect enantioseparations in ways that are not fully understood yet, for the lack of systematic studies. This makes chiral analysis of some pharmaceuticals difficult to reproduce. Once a column has been exposed to a modifier, the selectivity of certain pairs of enantiomers may change, for the better or the worse. We study the behavior of five enantiomeric pairs, three which are highly sensitive to the addition of certain modifiers and two that have little sensitivity to these modifiers. Their use permits the determination of the extent of the memory effect response on individual columns. The selectivity of 4-chlorophenylalanine methyl and ethyl ester, and of ketoprofen improve as a solution of ethanesulfonic acid is percolated through the column. As a result, these pairs are most useful for the determination of the extent of acid memory effect on a column. The selectivity of propranolol HCl and, to a lesser degree, Tröger's base increases as a solution of diisopropylethylamine is percolated through the column. The separation of each one of these five pairs is inversely affected by the percolation of the opposite acid/base solution. We used *trans*-stilbene oxide (TSO) as a 'standard' to determine the column stability because no memory effect is observed for it (its retention, enantioselectivity, and resolution remain constant). Understanding whether a column is under the influence of the memory effect is critical to both the analysis of pharmaceutical ingredients and to the development of preparative purification techniques for racemic mixtures. Thus, columns that were unreliable for method development and method transfer, due to the memory effect and a lack of proper solvent exposure records, can now be used.

1. Introduction

The pharmaceutical industry relies on HPLC analysis as one of the most suitable systems for quantitative analysis [1]. Numerous laboratories are involved with bringing new active pharmaceutical ingredients (API) to the market place, which requires the transfer of methods between columns, instrumentation, and laboratories [2]. The transfer and scaling of a method developed on one column to a second column depends on the proper reproducibility of the mobile phase composition, the flow rate, the mass of stationary phase, and the sample injection mass. Additionally, the variability in manufacturing batches of stationary phase can influence the separation when transferring a method to a second column. In this research, the problem of batch variability was minimized by using one column from each batch of stationary phase. The most significant consideration when transferring a separation method developed on the amylose tris(3,5-dimethylphenylcarbamate) (CHIRALPAK® AD®, Diacel Industries, Osaka, Japan) column to other columns of the same stationary phase is a phenomenon called the memory effect, first studied by Ye and Stringham in 2001 [3,4]. Once a column with this stationary phase has been exposed to an acid or a base mobile phase modifier, the separation of certain, but not all, racemic mixtures will change. After removing the mobile phase modifier, the change in separation capacity is retained during the percolation of the mobile phase through the column for thousands of column volumes [5].

In this research, the mobile phase composition was kept constant and the flow rate for each column was adjusted to have identical retention times of the solvent peak, even on columns with different dimensions. The injection mass was adjusted to give the same ratio of enantiomeric mass to stationary phase mass for each column. For example, the amount of *trans*-stilbene oxide (TSO) enantiomers injected on a 4.6 × 150 mm analytical column (labeled 4019) was 10 μL of a 1 mg/mL solution, giving an injection mass of 10 μg/1.55 g of stationary phase. To provide the same injection mass to stationary phase mass ratio, the injected mass on the 10 × 100 mm SMB columns (columns labeled SMB-C and SMB-E) requires injecting 2.6 times as much material on the SMB columns, due to the extra stationary phase in the larger SMB columns. As a

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result, the injection volume on the SMB columns was 26 μL at a concentration of 1 mg/mL.

A better understanding of the memory effect, as well as efficient tests for the detection of this phenomenon, are crucial to the separation of racemic mixtures. By focusing on a stationary phase that clearly exhibits this phenomenon, three objectives can be accomplished. First, understanding the correct method for detecting the memory effect is critical for developing methods for controlling the phenomenon, thus allowing the separation of additional racemic mixtures on this stationary phase. Second, properly determining if this phenomenon exists on other carbamate stationary phases will expand the number of racemic mixtures separated by chromatographic methods. Third, using a column exhibiting a known memory effect in preparative separations can be combined with partial asymmetric synthesis and/or enantiomeric enrichment crystallization to improve success in purifying new APIs.

In order to apply the memory effect properly to the development of analytical and preparative methods, a decisive test must be developed to determine whether a column has been exposed to mobile phase modifiers. Determining if a column has been exposed to a modifier and whether that column is still under the influence of the same modifier can make the difference between success and failure in separating a racemic mixture. The goal of this research was to determine which racemic mixtures are good test probes for the memory effect and to determine if one or more steady-state conditions exist within the memory effect phenomenon.

2. Experimental

2.1. Chemicals

The mobile phase used in the following experiments consisted of hexanes obtained from Fisher Scientific (Pittsburgh, PA, USA) and manufactured by JT Baker (Phillipsburg, NJ, USA). This product contains more than 85% *n*-hexane, with less than 2% methyl-cyclopentane and small amounts of branched hexanes. The alcohol modifier of the hexanes was ACS reagent grade alcohol containing 90% ethyl alcohol, 5% isopropyl alcohol, and 5% methyl alcohol. Chemicals obtained from Sigma–Aldrich (St. Louis, MO, USA) included 4-chlorophenylalanine methyl ester (4CPME), 4-chlorophenylalanine ethyl ester – 97% (4CPEE), 1,3,5-tri-*tert*-butylbenzene – 97% (TTBB) used as a column void marker, ethanesulfonic acid – 95% (ESA), propranolol hydrochloride – 99%, and Tröger's base. The ketoprofen – 99% was obtained from Spectrum Chemicals (New Brunswick, NJ, USA), and the *trans*-stilbene oxide – 97% was obtained from Acros Organics. The *N*,*N*-diisopropylethylamine (DIPEA) was obtained from Alfa Aesar (Ward Hill, MA, USA).

2.2. Equipment

An HP 1100 (Agilent, Santa Clara, CA US) was used to carry out all the experiments and to collect all the measurements reported. A single pump and a single batch of prepared mobile phase were used to eliminate possible variations of the ethanol concentration during individual tests. A column heater was used to control the separation temperature at 40 °C. An autosampler was used to allow for repetitive injections over the entire data collection period. A single wavelength detector was used, all the racemic mixtures tested providing an excellent signal to noise ratio at 210 nm.

2.3. Columns and stationary phase

The only analytical 4.6 × 150 mm column used for these studies was packed by Chiral Technologies (West Chester, PA, USA) and was labeled 4019. This column had been used in previous studies of the memory effect but had not been exposed to any mobile phase or additive other than those which were documented in a previous publications [5,6]. Specifically, this column was exposed to ESA, ethanol, DIPEA, and hexanes as mobile phases and/or additives and also to the racemic mixtures of TSO, 4CPEE, 4CPME, ketoprofen, propranolol, and Tröger's base. Prior to exposing this column to mobile phase modifiers the column was tested with the six racemic mixtures. The selectivity and resolution data collected from these initial tests have been recorded and used as a control value labeled '4019 – original'.

Two preparative 10 × 100 mm columns were also packed by Chiral Technologies, these columns were labeled SMB-C and SMB-E. These two columns had been used previously for the preparative separation of Tröger's base. All solvents used in these columns had been reported by Mihlbachler et al. [7]. In particular, the columns were exposed to methanol, isopropyl alcohol, and Tröger's base. Additionally one of these columns (labeled SMB-C) had been used for the preparative separation of 4CPEE, 4CPME, ketoprofen, Tröger's base, propranolol, and TSO. The SMB-C column was additionally exposed to both ESA and DIPEA.

Two additional 4.6 × 250 mm columns were obtained from Chiral Technologies and labeled ID006 and FB001. These columns had been used by numerous groups and laboratories. Due to their unknown solvent history, these columns were excellent for comparisons to the previous columns listed. By comparing the separation of different racemic mixtures on these columns with unknown solvent histories, we could determine whether they had been exposed to additives inducing the acid memory effect (AME) or base memory effect (BME).

3. Procedures

The mobile phase was made as 4 L of 90/10 (v/v) hexanes/ethanol, to ensure that all columns were exposed to the same mobile phase and that all separations would use the same mobile phase. All samples (4CPEE, 4CPME, ketoprofen, propranolol, Tröger's base, and TSO) were made at a concentration of approximately 1 mg/mL in a solution of 90/10 (v/v) hexanes/ethanol. Each column was kept at a temperature of 40 °C when in use. The flow rate was controlled for each column to ensure that the TTBB, used as a column void marker, eluted at the same time from each column.

Before any samples were injected on to a column, the column was flushed with mobile phase for at least twenty column volumes. In the case of the analytical 4.6 × 150 mm column (4019), this mobile phase volume was 30 mL. For the preparative 10 × 100 mm columns (SMB-C and SMB-E) this mobile phase volume was 80 mL. For the two 4.6 × 250 mm columns with unknown history (ID006 and FB001), an additional step of flushing with isopropyl alcohol was carried out prior to the hexanes/ethanol flush. This additional flush was to ensure that the hexanes/ethanol mobile phase was compatible and miscible with the previous (unknown) mobile phase held within the column when received.

The injection sequence followed for each column was: 4CPEE, 4CPME, ketoprofen, propranolol, Tröger's base, and then TSO. This sequence was repeated three times for each column.

The determination of whether a column had been exposed to the additives inducing AME or BME was done by comparing the selectivity of all six racemic mixtures to data collected previously [5]. An example of these data and the concept is presented in Fig. 1 These data were collected by exposing the analytical 4.6 × 150 mm column (4019) to a maximum load of ESA, followed by the continuous injections of the six racemic mixtures made until the selectivity of the 4CPEE and ketoprofen reached a value of one. Then, the column were exposed to DIPEA, and the injections of the six racemic mixtures was continued until the selectivity of Tröger's base and

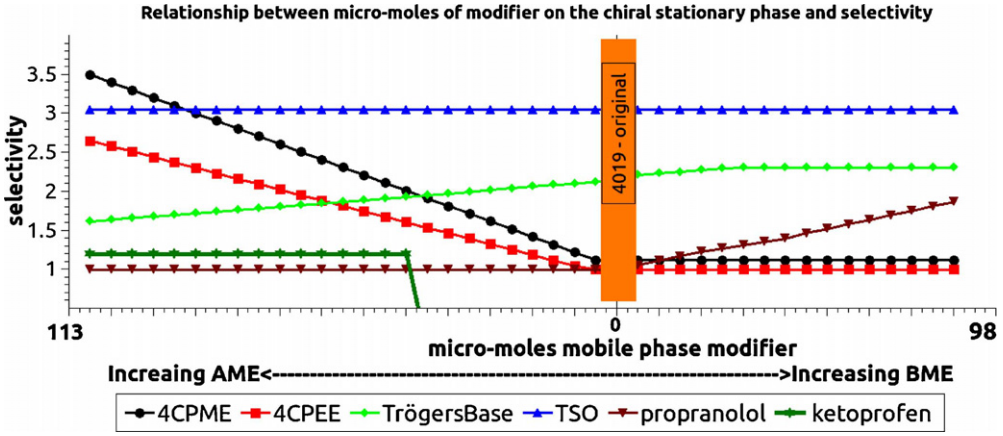

Fig. 1. Using the information collected by testing the specific selectivities of the 4-chlorophenylalanine derivatives, Tröger's base, the TSO, the propranolol and the ketoprofen racemic mixture, a finite region could be determined for how each column responded as a specific amount of either acid or base mobile phase was exposed to the stationary phase. The far left number of 113 micro-moles (μmol) is derived from the maximum mass of ESA injected onto the column to create the acid memory effect. The far right number of 98 μmol is derived from the maximum mass of DIPEA injected onto the column to create the base memory effect. The individual selectivity curves generated for this conceptual representation were collected from previous articles on the memory effect [5]. Since the selectivity data collected on the 4019 column when it was received represents a column with no history of exposure to mobile phase modifiers these selectivities measured on this column are labeled as a region near 0 μmol of mobile phase modifiers. Condition for all columns: 1 mL/min, 40 °C, 90/10 (v/v) hexanes/alcohol mobile phase.

propranolol reached a maximum value. In this figure, the *x*-axis is represented by the extent of the memory effect measured on a column which can be related to the number of moles of mobile phase additive adsorbed on the surface of the stationary phase. In estimating the far left *x*-axis limit, the maximum amount of ESA injected on to the stationary phase was measured numerous times, with the largest molar amount being 113 micro-moles (μmol) by sequential 50 μL injections of ESA at a concentration of 10.08 mg/mL. Additional injections of ESA did not change the selectivity or resolution of the six racemic mixtures. It is important to note that this number of 113 μmol in not the actual amount of material adsorbed on surface due the unlikelihood of the entire injection being retained. In determining the far right *x*-axis limit, the total number of injections of the base mobile phase additives to achieve the maximum base memory effect did not require more than twelve 100 μL injections of DIPEA at a concentration of 10.21 mg/mL (98 μmol of DIPEA). The solid rectangles represent a region of separation capacity related to the selectivity of the six racemic mixtures eluting from each column. A broad region has been used to incorporate numerous variable related to measuring selectivities: variations in the mobile composition, in injection concentration, in temperature, and in the unknown mass of mobile phase additive adsorbed on the stationary phase. Further experimentation is required to determine the total amount of mobile phase modifier adsorbed on the surface of the stationary phase.

4. Results and discussion

4.1. The analytical 4.6 × 150 mm column

Prior to exposing the 4.6 × 150 mm column (labeled 4019) to any mobile phase modifiers, injections of all six racemic mixtures were performed onto the column. Fig. 2a shows the whole set of results acquired regarding the selectivity (4019 – original). Fig. 2b shows the resolution of the five racemic mixtures eluted from this column (4019 – original). As expected, the two standards – Tröger's base and TSO – were the only racemic mixtures that were fully resolved on this column. Partial resolution was observed for 4CPME and propranolol, while the 4CPEE was not resolved at all and the ketoprofen did not elute from the column. These results are considered to be conventional analytical results on any new CHIRALPAK AD column [8]. It has been shown previously that resolution of the enantiomers of 4CPEE, ketoprofen, and 4CPME indicates that the column is under the influence of the AME [5]. It has also been shown that full resolution of the propranolol enantiomers indicates that the column is under the influence of the BME [5]. Fig. 1 shows a graphical analysis of these results. In the graphical analysis, the *x*-axis indicates the degree of memory effect that a column exhibits. The far left is the maximum AME while the far right is the maximum BME. The rectangle labeled '4019 – original' uses the selectivity data collected on the 4019 column when it was received and represents a column

Table 1
The selectivity (α) and resolution (R_s) results of the six racemic mixtures on five columns.

Column ID	TSO		4CPEE		4CPME		Ketoprofen		propranolol		Tröger's base		Presence of memory effect
	α	R_s	α	R_s	α	R_s	α	R_s	α	R_s	α	R_s	
Original[a]	2.78	5.05	1	0	1.09	0.5	0	0	1.21	0.72	2.37	3.4	None
4019[b]	2.9	5.15	1	0	1.06	0.49	0	0	1.3	0.94	2.25	3.15	None
SMB-C[c]	2.63	4.27	1.67	1.68	1.94	2.27	1.2	0.67	1.1	0.4	1.83	1.85	AME
SMB-E[d]	2.56	5.19	1	0	1.06	0.33	0	0	1.53	1.72	2.31	2.22	BME
ID006[e]	2.7	4.31	1	0	1.08	0.39	0	0	1.38	1.04	2.33	2.95	BME
FB001[f]	2.86	4.25	1.36	1.19	1.57	1.85	1.27	0.96	1	0	2.37	2.86	AME

[a] The original analytical 4.6 × 150 mm column test results when it was initially received.
[b] The original column after exposure to both acid and base mobile phase modifiers.
[c] The 10 × 100 mm use in preparative studies.
[d] The 10 × 100 mm column only used in the study by Mihlbachler et al. [7].
[e] One of the 4.6 × 250 mm columns with unknown solvent and mobile phase modifier exposure.
[f] The second 4.6 × 250 mm column with unknown solvent and mobile phase modifier exposure.

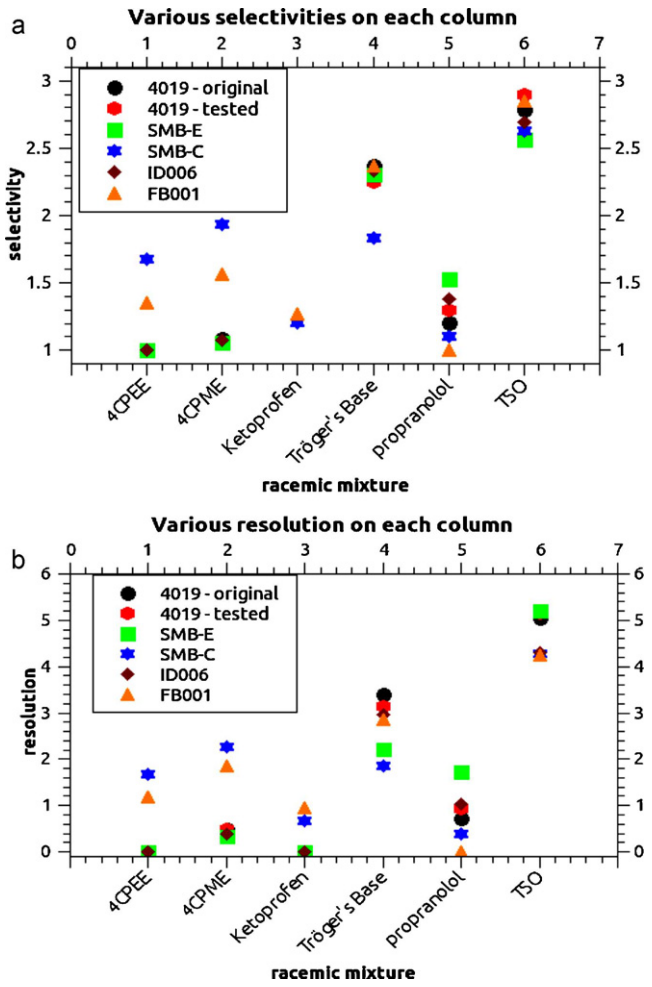

Fig. 2. (a) The selectivity measurements for the five columns using the six racemic mixtures. SMB-C had the largest changes in separation capacity. Only the selectivity of the TSO racemic mixture was similar to the 4019 column results. Similarly the FB001 column did not perform as the original column in the separations of these enantiomers. Changes in the separation of the ID006 and SMB-E column can be seen in the selectivity of the propranolol and Tröger's base enantiomers. (b) Similar to the changes seen in the selectivities, the resolution measured on the SMB-C and FB001 showed specific changes in the capacity to separate the racemic mixtures which required acidic mobile phase modifiers. The resolution of the propranolol and Tröger's base on the SMB-C column is significantly different than the original 4019. Condition for all columns: 0.8 cm/s linear velocity, 40 °C, 90/10 (v/v) hexanes/alcohol mobile phase.

with no history of exposure to mobile phase modifiers. The edges of the rectangle represent the selectivities measure on this column as a region near 0 μmol of mobile phase modifiers. Additionally, the actual selectivity and resolution values for each racemic mixture have been recorded in Table 1.

The rectangle labeled 4019-tested in Fig. 3 shows the results of exposing the 4019 column to either acid or base mobile phase modifiers, which occurred during the previously mentioned documented experiments. The elution of the 4CPEE enantiomers was similar to the 4019 column, and no separation was detected. The separation of the 4CPME was nearly eliminated compared to the original data. At the same time the separation of the propranolol enantiomers improved slightly. This would indicate that the chiral environment had changed and was more likely to recognize other racemic mixtures similar to propranolol. These slight changes in selectivity might indicate that this column was under the influence of the BME, yet the change was not large enough to fully resolve the propranolol racemic mixture. Fig. 2a shows the graphical display of this slight change in the selectivity (4019 – tested) while Fig. 2b shows the graphical display of the resolution for these racemic mixtures (4019 – tested). In Table 1 the actual selectivity and resolution values have been recorded.

4.2. The preparative 10 × 100 mm columns

Two preparative columns were used in this study. One column (labeled SMB-E) was only exposed to methanol, isopropyl alcohol, and Tröger's base prior to this study. The second preparative column (labeled SMB-C) was additionally exposed to hexanes, ethanol, ESA, DIPEA, 4CPEE, 4CPME, ketoprofen, propranolol, and TSO prior to this study. The variations in the selectivity measured using the 4019 original column (Fig. 2a) and each preparative column (Fig. 2a – SMB-E and SMB-C) are due to each column being exposed to different solvent mobile phase modifiers prior to the injections performed in this study.

The column labeled SMB-E exhibits two significant changes from the original separation selectivity of column 4019. In Fig. 2a and b (SMB-E), the propranolol enantiomers were fully resolved while the 4CPME enantiomers had little selectivity, indicating that the column was under the influence of the BME. Neither the TSO nor the Tröger's base showed any significant changes in selectivity or resolution, and as a result, these racemic mixtures cannot be used to detect the presence of the BME. Fig. 3 (SMB-E) shows the rectangle representation of the selectivities measured when this column exhibited the BME condition. The actual recorded selectivity and resolution for the six racemic mixtures tested on this column are shown in Table 1.

The column labeled SMB-C shows three significant differences from the original selectivity data collected on column 4019 and column SMB-E (see Fig. 2a and b – SMB-C). The most significant change was the elution and chiral recognition of the ketoprofen enantiomers. Additionally, both enantiomers of the 4-chlorophenylalanine derivatives were fully resolved. The last difference observed was that the propranolol enantiomers were barely separated. Neither the TSO nor the Tröger's base showed any significant change in selectivity or resolution, and as a result, these racemic mixtures cannot be used to detect the presence of the AME. These results would indicate that this column was under the influence of the AME. This column was not at the maximum AME as described in numerous previous articles about this phenomenon. Instead there was a residual influence on the separation of certain racemic mixtures. The rectangular depiction of the selectivities measured which indicate the AME is present can be seen in Fig. 3 (SMB-C). The actual selectivity and resolution data for this column are recorded in Table 1.

4.3. The 4.6 × 250 mm columns with unknown solvent exposure

The two 4.6 × 250 mm columns with unknown solvent exposure were labeled ID006 and FB001. All previous data concerning their exposures to mobile phase and mobile phase modifiers were either lost, not recorded, or not provided. As a result, the chiral environment inside the columns was unknown. The separation of the TSO enantiomers indicated that the stationary phase had not been damaged (see Fig. 2a and b – ID006 and FB001).

Fig. 2a shows the selectivity of five of the racemic mixtures separated on the ID006 column. The ketoprofen enantiomers did not elute from this column. The 4CPEE enantiomers did not separate, and the 4CPME enantiomers had only a slight separation. The propranolol enantiomers had a larger selectivity on this column than on the original 4019 column (Table 1). Fig. 2b shows that the resolution of these six racemic mixtures indicates that the separation of the propranolol enantiomers on this column is slightly improved. From these results, it should be recognized that this column may have been exposed to basic mobile phase modifiers, but separates

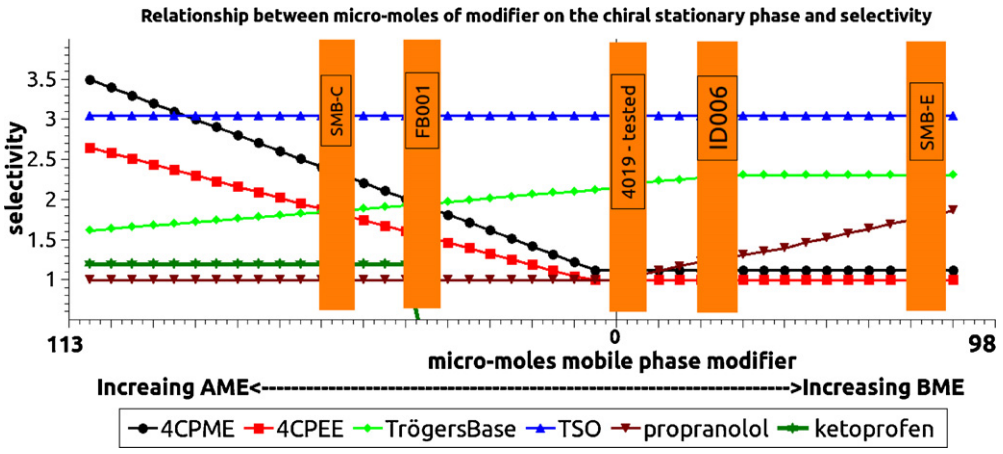

Fig. 3. Even after exposure to acid and base mobile phase modifiers the 4019 column performs similar to the original conditions of the column with only a slight decrease in the selectivity in the separation of the 4CPME and an increase in the separation selectivity of the propranolol mixture. The SMB-E column separated the propranolol similar to a column that has been exposed to a base mobile phase modifier, yet the column has no history of this occurring. Similarly, the ID006 column has the capacity to separate the propranolol slightly better than the original stationary phase. The SMB-C column has been exposed to mobile phase modifiers and still retains the capacity to separate mixtures that require an acid mobile phase additive. Similarly, the FB001 column has some capacity to separate mixtures requiring an acid mobile phase. Condition for all columns: 0.8 cm/s linear velocity, 40 °C, 90/10 (v/v) hexanes/alcohol mobile phase.

mostly the enantiomeric mixtures similar to a new column. Fig. 3 (ID006) shows the rectangular region representation of the extent of BME seen on this column. The actual selectivity and resolution data for this column are listed in Table 1.

Fig. 2a illustrates the selectivity of all six racemic mixtures separated on the FB001 column. The most recognizable change was the elution and separation of the ketoprofen enantiomers. The second clearest change from the original column was the retention of a single peak for the propranolol enantiomers. Additionally, both 4CPEE and 4CPME were fully resolved (see Fig. 2b – FB001). The selectivity and resolution data (shown in Fig. 2a and b) indicate that this column was under the influence of a residual AME. The rectangular representation of this memory effect is shown in Fig. 3 (FB001). The actual selectivity and resolution data for this column are listed in Table 1.

5. Conclusions

The goal of this research was to identity a method allowing the determination of whether a column is under the influence of a memory effect. The use of the enantiomers of 4-chlorophenylalanine derivatives, ketoprofen and propranolol demonstrated that certain columns do have the capacity to separate these racemic mixtures differently than a recently purchased column. As a result, a simple test was devised and implemented. This test successfully showed that the separation of the 4-chlorophenylalanine derivatives could indicate which columns have been exposed to AME mobile phase modifiers. The separation of the ketoprofen enantiomers did not provide as much information since the separation of these enantiomers occurs only for a specific period of time after the column was exposed to an acidic modifier. Yet, the analysis of a ketoprofen racemic mixture is a cheap and easily available test probe compared to the 4-chlorophenylalanine derivatives. The propranolol enantiomers did quite well at demonstrating that a column had been exposed to a BME mobile phase modifier. However, both the Tröger's base and the TSO are poor probes for detecting a memory effect. The separation of Tröger's base returns to a modifier naive condition rapidly, and separation of the TSO is completely unaffected by previous exposure of the stationary phase to any modifiers.

The tests of the six columns studied did indicate that at least four different steady-state conditions do exist within the memory effect phenomenon. The first is that of recently purchased columns under mobile phase modifier naive conditions, older columns that have not been exposed to mobile phase modifiers, and columns that have been returned to the original separation condition. A second, BME steady-state condition also exists, which can be reached and easily kept if the column is not exposed to significant concentrations of acidic modifiers. At least two steady-state conditions exist with a capacity to separate enantiomers that require an acidic mobile phase modifier. Two of the columns tested in this research show different levels of capacity to recognize certain racemic mixtures. Under one steady-state condition, all three racemic mixtures were separated with appropriate selectivity. This steady-state condition may be more of a saddle point instead of a stable state. The second acid steady-state condition was created by exposing a column to an acidic mobile phase modifier which can separate the 4-chlorophenylalanine derivatives but not the ketoprofen racemic mixture. Yet, the lack of a separation of propranolol enantiomers can detect this steady-state condition. For this reason, the enantiomers of ketoprofen and propranolol can easily be used to determine the presence of the four steady-state conditions detected in this research.

The shape of the peaks that are eluted upon injections of both ketoprofen and propranolol on a CHIRALPAK AD column can determine the presence of the memory effect on the column. If ketoprofen eluted and its enantiomers are separated, the column in question has been exposed to an acidic mobile phase modifier, as this is one of the AME steady-state conditions. If the ketoprofen eluted within 30 min with only a single broad peak, the column is in the weaker AME steady-state condition. If the ketoprofen does not elute from the column, the propranolol peak shape can determine two additional steady-states. If the propranolol enantiomers are completely resolved with full selectivity, this column has been exposed to a base mobile phase modifier and this is the strong BME steady-state condition. If the propranolol racemic mixture eluted with only a slight selectivity (below 1) then this column should be considered to be mobile phase modifier naive, this is the original condition and is the fourth steady-state condition.

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