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Case Report

A Step Towards Efficient Clinical Diagnosis: Monolithic Silica particles Modified with Polystyrene as an Efficient Stationary Phase in Liquid Chromatography

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Abstract

The extra-modified protocol for the synthesis and modification of partially porous silica monolith particles into polystyrene bound partially sub-1µm silica monolith particles, selection of proper column dimensions, the modified packing procedure, has made it possible to achieve very good separation media for the analysis of small molecules in HPLC. The catalyst assisted ligand binding and RAFT polymerization was good enough for the attachment of uniform polymer film on to the silica particles. The column packed with those particles was outstanding in the achievement of high separation efficiency (230,200 plates/m), better than the separation efficiency of column packed with core shell particles. The column of this study in conjunction with mass spectrometer could be a handsome approach in the analysis of small molecules especially for proteomic and glycomic analysis.

Keywords: Extra-ordinary Separation Efficiency; Evaluation Via HPLC; Polystyrene Modified Silica Monolith Particles; Partial Monolith Architecture

Abbreviations

BET: Brunauer-Emmett-Teller; BJH: Barrett- Joyner-Halenda; FE-SEM: field Emission Scanning Electron Microscopy; FE-TEM: field-Emission Transmission Electron Microscopy; HETP: Height Equivalent to a Theoretical Plate; RAFT: Reversible Addition-Fragmentation Chain Transfer; TMOS: Trimethylorthosilicate.

Introduction

Advanced separation, quantification and analysis of complicated samples owe their existence to HPLC and advancement in stationary phases used in HPLC in current day research [1-4]. The fast track growth in the fields of clinical, bio analytical, natural, and pharmaceutical, sciences has made it surly challenging to analyze the complex mixtures with good statistical backgrounds [5-7]. This surprisingly led the column technology in RPLC to the advanced level of research. The literature about the involvement of the Porous-non-porous silica both in the form of bare and modified particles have been widely used as separation tool in separation and solid phase extraction [8-12]. Surfaces of the metal-oxides are converted to different materials and those substance find a lot of applications in variety of fields [13-18]. The highly efficient nature associated with tine particle size was reported in the introductory literature [19-21]. To the great extent the core-shell particles and very small particles which are porous play the key role. To see the articles related with decreasing particles dimensions see Ref. [22-31]. To get the desired better selectivity out of the partially porous silica monolith media in contrast to the core-shell media we have been trying to since long to bring forward the ideas of better packing along with incorporation of the small particle [29-34]. Looking back at our previous article we achieved tiny particles as separation media being functionalized with C18 [31] but we had the problem of packing related to those particles which resulted in

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the frit clogging while packing. The sub-1 μm silica particles have been changed into polystyrene incorporated phase parallel to the adaptation of a good packing technique. The sedimentation phenomenon being given in the Ref. [34] was executed in a little technical way to achieve particles in the narrow range and to eliminate the very tiny particles. The sedimentation process was carried out by making suspension of the particles in methanol/water (90/10) for silica monolith particles and 100 % methanol for polystyrene bound phase using shaker for about 5 min. The suspension was kept in position undisturbed for some time and the supernatant was removed by means of spatula. During the suspension process the averaged sized particles settled down to the bottom while the minute particles within the supernatant were removed. The sedimentation process was repeated five times. The packing protocol was modified along with modifications in the slurry packer which has resulted in better bed packing inside the column with minimum void volume leading to fast mass transfer kinetics. The successful modification of tiny particles with sub-1 μm size was exhibited in this study [29,30]. The finally obtained stationary phase was packed in stainless steel column (1.8 mm × 300 mm). Although we had some problems in packing since the sticky polystyrene modified particles of small particle size remain aggregated with each other but that problem was solved by the adaptation of sophisticated washing protocol with various solvent combination. The physically aggregated particles were separated during the slurry preparation step before packing by means of sever agitation and vortex which was repeated five times [29,30]. Each time fresh methanol was added and after agitation the suspension was kept in position for some time and the supernatant including the minute particles was removed. The column resulted in very high separation efficiency of 230,200 plates/meter for the separation of five benzene derivatives in addition the column was capable of fast HPLC analysis. The N-value (Ca. 230,200 plates/m) for the column of current study is very high than that (188,000 plates/m) of the column of previous study [30]. Along with improved packing, silica monolith particles of current study have large pore size (368 Å) and we expect high mass transfer kinetics using large pore sized stationary phase. Production scale of silica monolith was improved 4 times compare to our previous study [31].

Experimental

Reagents and materials

The chemicals reagents, from Sigma-Aldrich (St. Louis, MO, USA): acetic acid, anhydrous toluene, trimethylorthosilicate (TMOS), polyethylene glycol (PEG) 10,000, chlorodimethyloctadecylsilane, hexamethyldisilazane, chlorotrimethylsilane, urea, and styrene.

Mallinckrodt Baker (Phillipsburg, NJ, USA): HPLC grade methanol, acetonitrile, acetone, and water were obtained from. Valco (Houston, TX, USA). Screen frits (1.6 mm diameter, 0.08 mm thickness, and 0.5μm pore size) were purchased from Glass lined stainless steel tubing (15 cm, 1.8 mm ID, 1/8 inch OD) and silica capillary (50μm ID, 365μm OD) were purchased from Grace (Deerfield, IL, USA).

Ground silica monolith particles synthesis

Partially porous sub-1 μm silica particles were prepared by the delicately controlled sol-gel process. The volume of TMOS (15 ml) and catalyst (80 mL 0.01 N acetic acid), was altered in comparison to the amount of PEG (8 g) and urea (8.4 g). The solution was consistently shake in low temperature environment for little less than one hr. The solution was kept in LC-oven at 40˚C for 60 h followed by heat treatment at 120 ˚C in GC-oven in Teflon lined autoclave for 50 h. The resultant gel was smashed up with spatula so that the upper water layer and gel were homogeneously mixed into a viscous suspension followed by drying at 70˚C for 15 h. The silica monolith particles were sedimentation treated five times using methanol/water (90/10). The silica monolith particles were then dried at 80 ˚C for 10 h.

Initiator attached silica monolith particles

A little changed formulation was used in current study than those of the Ref. [29,31]. 4-Chloromethylphenylisocynate is not completely miscible in toluene. Thus xylene was used along with small amount of 4-methyl-2-pentanone as solvent mixture in current study. 250 mg of incoming halogen moity and 100 mg of the sponsoring specie DBTDC were used in the mixture containing (25 ml xylene + 5 ml 4-methyl-2-pentanone). The reacting reagent were sonicated for about 25 min. The high temparatue treated silica particles (1000 mg) were kept dried at elevated temperatures for 2~3 h. After 4-CPI attachment the silica particles were washed using solvents such as toluene and acetone followed by filtration and air drying. A sonicated solution of reaction mixture containing 600 mg of sodium diethyldithiocarbamat in 25 mL anhydrous THF was N_2-purged for 10 min followed by the addition of 4-CPI bound silica particles and the entire reaction mixture was refluxed at 55 ˚C overnight under stirring condition. The so far obtained particles were inculcated in series of washing units, using the THF, the MeOH/water (80/20 v/v %) and the acetone and was stored in the vacuum desiccator. For reaction scheme see figure 1 in Ref. [30]. Styrene envolved RAFT polymerization mechanism was used for the immobilization of the polymer upon the particles. The particles thus obtained were suspended in the 30 mL anhydrous100 mL toluene which were refluxed at the boiling point of the solvent being used as the media for the reaction for 15 h. Styrene (3 mL) and anhydrous toluene (3 mL) were added drop wise via the dropping funnel. The solution of reagen was shaked well in the N_2-environment during the course of reaction. The styrene bound silica particles being washed with toluene, 2-propanol, 2-propanol/water (95/05) and acetone and stored in desiccator. Reaction scheme being given in figure 1 in Ref. [30].

Figure 1: FE-SEM images of bare (A and B) and polystyrene modified silica monolith particles (C and D) where A and C are wide views while B and D are close views. Y is the microscopic view and Z is the TEM image of the modified silica monolith particles. Magnification scale of A and C is 10 μm, B and D is 1μm and for Z it is 20 nm.

Column packing for evaluation in HPLC

Column assembling and packing of the column was carried in accordance with the procedure published in our previous article Ref. [29].

Results and Discussion

Characterization

HITACHI S-4200 (Tokyo, Japan) was used for taking field emission scanning electron microscopy (FE-SEM) images of the modified and unmodified silica monolith particles while the TEM images were obtained using JEOL JEM2100F (Tokyo, Japan) field emission transmission electron microscopy (FE-TEM). BET (Brunauer-Emmett-Teller)/BJH (Barrett- Joyner-Halenda) nitrogen adsorption/desorption isotherms, BJH pore sizes, and BET specific surface areas were measured using BEL-Japan (Osaka, Japan) BELSORP-Max. The carbon load data was measured by a Thermo Electron elemental analyzer Flash EA1112 (Waltham, MA, USA). A Malvern Master Sizer 2000 (Worcestershire, UK) particle size analyzer was used to get particle size distribution.

Architecture and morphology of the stationary phase

The morphology of polystyrene co-polymer film on the surface of silica monolith particles is visualized in Figure 1. Polystyrene modified particles are well dispersed as seen in the microscopic view (Y) of Figure 1 while the sub-1 μm particle size can also be estimated from the images in Fig.2. The exact particle size has been find out by particle size distribution. We can observe that the particle surface after modification (Figure C and D) is smoother than that of the bare silica particles (Figure A and B) due to the formation of polymer film. The visible light dark spots in the FE-TEM images of polystyrene modified silica particles indicate pores structure which seems to be projected in dark irregular spots. Pores can also be observed in the TEM image (Figure 2Z) which seems to be quite large and were confirmed by the nitrogen adsorption/ desorption analysis.

Particle size distribution

The number based particle size distribution data for bare and modified silica monolith particles is very close to that of Ref. [31], see figure 3A and 3B respectively table 1. Sedimentation process [31] was carried out to remove the fine particles both before and after modification. The sedimentation process was repeated three times both for unmodified and modified silica monolith particles. Thus the overall average particle size was confined to a narrow range. A little more increase in particles size after modify cation in comparison to the previous study [31] is due to the immobilization of polystyrene co-polymer film in current study while in previous study C18 was bounded on to the surface of silica monolith particles. The difference in the effect of C18 and polystyrene co-polymer film onto silica particles can be observed from the FE-SEM images of previous [31] and current studies where the surface of polysty-

Figure 2: Particle size distribution before (A) and after (B) modification of silica monolith particles. Particle size of unmodified and modified silica monolith particle at d (5) is 0.894 µm and 1.234 µm respectively.

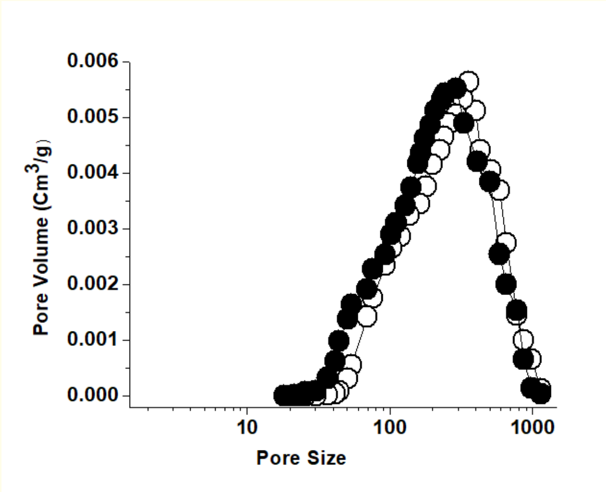

Figure 3: BJH- pore size distribution data (Å) for the unmodified (o) and polystyrene modified (●) silica monolith particles.

rene modified particles of current study is smoother than that of the C18-modified silica particles of previous study [31]. The confinement of polystyrene modified silica monolith particles to a narrow range is critical for the improvement of packing quality. The removed fine particles were collected and they were subjected to further experimentation for other applications. Some of the polymer film are attached to the inner surface of pore located within the bulk of silica particles not contributing to the overall increase in the particle size but do contribute to decrease in the pore size of silica particles after polystyrenemodification. The decrease in pore size and pore volume and corresponding changes in the surface area has been given in the section of pore size distribution.

	Bare Silica monolith particles			Polystyrene modified silica monolith particles		
	d(0.1)a	d(0.5)b	d(0.9)c	d(0.1)a	d(0.5)b	d(0.9)c
Number based	0.59	0.85	1.86	0.68	1.03	2.15

Table 1: Number based particle size distribution for bare and polystyrene modified silica monolith particles after the removal of fine particles in µm unit.

Particle diameter corresponding to the integrated area ratio of

a) 0.1 when integrated in the range of 0-d diameter.

b) 0.5 when integrated in the range of 0-d diameter.

c) 0.9 when integrated in the range of 0-d diameter.

Critical analysis of pore size for bare and polystyrene bound silica monolith particles

In current study the catalyst assisted polystyrene modification of silica monolith particles along with decreased particle size and increased pore size has been illustrated for the highly improved chromatographic performance of the stationary phase. The BET/BJH pore size analysis (Figure 4) given in table 2 shows that average pore size of the silica particles of current study is very close to that of the previous study [31]. While the average pore size of polystyrene modified silica particles of current study is smaller than that of the C18-bound silica particles of Ref. [31] since the catalyst assisted RAFT polymerization results in the formation of homogenous and uniformly distributed thin polymer film [30]. While the C18-modified silica particles have relatively large pore size after modification due to the attachment of C18 chains instead of polystyrene polymer film. The BET/BJH analysis (Table 2) shows that the corresponding changes in pore size, pore volume and surface area of silica particles after modification in the current study are in agreement with each other. Having a look at the average pore size of bare silica and polystyrene modified silica particles in table 2 it can be seen that decreasing the size of silica particle results in the increased pore size. The formulations and duration of reaction steps involved in the synthesis of silica monolith particles have been technically controlled to get small silica particles with greater pore size. The conversion of urea to ammonia during aging process at 120 Cº is mainly responsible for the production of pores in silica monolith particles. Thus the relative amount of urea with respect to water, PEG, and amount of bulk silica gel is assumed to be critical for the determination of pore size in silica monolith particles. An overview of the pore size of silica particle out of the research work we have carried out in our lab reported in various journals is given in the table 2. We can see that the pore volume (cm^3/g) increases with increasing pore size, while the corresponding surface area ((m^2/g) decreases. As reported previously the formation of polymer layer in the deep pores results in the clogging of pore which are situated deep in the bulk of silica particles while the polymer chain form all around the pore on its inner surface are directed from the wall towards the center of the pore. These polymer chain spread and swell in the acetonitrile containing mobile phases and this swelling increases by increasing the proportion of acetonitrile in the mobile phase. The swelling of polymer chain results in fast mass transfer kinetics. Pores located on outer surface are not clogged. Thus the central portion of the silica particle becomes somewhat like solid core while the upper portion is porous exhibiting the pseudo-core/shell features [29,30]. This feature of the polystyrene modified stationary phase's accounts for the improved chromatographic performance. The possibility of the presence of nano pores in the polymer film may not be excluded since the surface area decreases by 14.4% from 118 m^2/g (silica particles) to 101 m^2/g (polystyrene modified silica particles) in comparison to the surface area which decreases by 24.54% that is from 110 m^2/g (silica particles) to 83 m^2/g for C18-bound silica particles [31]. In previous study [31] sub-1 µm C18-modified silica monolith particles were packed in 1.0 mm ID × 150 mm long stainless column while in current study we have used 1.8 mm ID × 300 mm long stainless column in order to avoid packing difficulty. Thus the wider ID column improved the packing quality because packing of small particles encounters packing problem. In current study along with successful modification of sub-1µm silica monolith particles with polystyrene the proper adjustment in the column dimensions has improved the overall performance of the resultant column. Small particle size and large pore size result in enhanced chromatographic performance. The column packing problem related with small particle size has been compensated by proper adjustment in the column dimensions. Whereas the problem of low carbon load related with small surface area (large pore size) is overcome by catalyst assisted polystyrene modification where polymer chain of uniform length [30] has been chemically bound to the silanol functionalities. The advantageous features of many strategies have been successfully availed in the current study which results in the overall enhancement of the chromatographic performance of the final stationary phase. The separation efficiency of the phase of current study which is 69,000 plates/column (230,200 plates/meter) is much better than the phase of the Ref. [30] which was 188330 plates/meter. Plate count/meter for the phase of current study is better than those of the commercially available stationary phases and is also better that those of the core-shell stationary phases. The column of current study result in fast HPLC analysis with analysis time of less than 2 min. Fast HPLC analysis is the need of the time in pharmaceutical companies since time is very critical factor in pharmaceutical analysis. Fast analysis in pharmaceutical companies is favored at the cost of high expenses. The factor contributing to fast analysis is the high permeability of the phase current study since improved permeability is the common feature of monolith stationary phases. The partial monolithic architecture of the phase of current study favor high influx of solvent during packing [29-31]. Lower column back pressure due to high permeability provides us the opportunity of high flow rates of the mobile phases leading to short analysis time. In addition to high separation efficiency, the phase of current study is cost effective and technically easy to prepare than the core shell particles. Thus the partially porous monolithic stationary phases seem to have the potential to take the LC to the next level of advancement.

Comparative elemental analysis between the phase of current study and those of the previous studies

The elemental analysis data being summarized in table 3 shows that the carbon load for the phase of current study is higher than that of the previous study [31], although the particle size, pore size and surface are of silica monolith particles in both studies are almost the same. This is due the fact that the polystyrene modified phase has higher carbon load than C18 phase since the polystyrene modified phase has longer polymer chains instead of the short C18 chain. The carbon load data for the phase of current study is close to that of the previous study [29] although the surface area (101 m^2/g) for the phase of current study is lower than that of the (131 m^2/g) of the previous study [29] owing to the small particle size of the phase of current study. The use of the catalyst (DBTDC) results in uniform and higher ligand density all around the silica particle which resulted in higher carbon load for the phase of current study which is close to that of the phase of sub-2 µm particle size [29]. Large size particles results in increased carbon load [35]. In one of our previous study [30] the carbon load (5.93%) was greater for the catalyst (DBTDC) assisted initiator bound silica monolith particles than the carbon load (4.30%) of the initiator bound silica monolith particles prepared without the catalyst. Second reason for the increased carbon load may be the polymerization [29] inside the pore of wide pore size (368 Å) in current study. The retention time span of all analytes is closely similar to that reported in Ref. [29] since the carbon load of both the phases are closer to each other. Thus the factors contributing to the higher carbon load of the phase of current study are a) polystyrene modification b) use of the catalyst c) large pore size of the silica monolith particles and d) technical control to successful accomplish various steps required for modification.

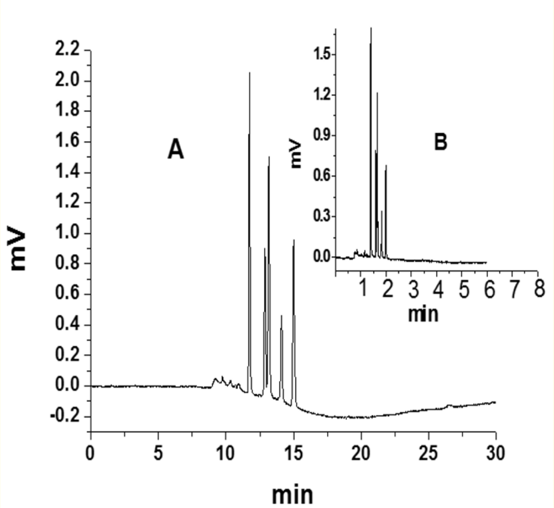

Figure 4: Chromatograms of five benzene derivatives (A and B) with elution order as phenol, acetophenone, 4-methyl-2 -nitroanailine, benzene and toluene from left to right. The chromatogram B is for fast HPLC analysis. 1.8 mm wide X 300 mm long stainless steel column was packed in current study where the detection wavelength was 214 nm. The flow rate of the mobile phase [65/35 (v/v) acetonitrile/water with 0.1% TFA] was 28 µL/min and 105 µL/min for chromatograms A and B respectively.

Effect of column dimensions on chromatographic performance and analysis with respect to packing quality

Dimensions of the column play a critical role in the overall chro-

	Bare silica particles				**Polystyrene bound silica particles**			**C18-bound phase**
	Previous studies			**Current study**	**Previous studies**		**Current study**	
	[35]	**[29]**	**[31]**		**[35]**	**[29]**		**[31]**
Pore size (Å)a	133	343	363	368	131	252	284	303
Pore volume (cm3/g)b	0.64	1.06	1.09	1.03	0.32	0.84	0.88	0.95
Surface area (m2/g)c	343	136	110	118	168	131	101	83

Table 2: The BET/BJH analysis results of the bare and modified silica monolith particles.

a) BJH adsorption average pore diameter.

b) Total pore volume at P/P0 = 0.99.

c) BET specific surface area.

Stationary phase	Current study 284 Å, partially sub-1 µm polystyrene bound silica monolith	Previous studies		
		polystyrene modified		C18-modified
		252 Å, partially sub 2- µm [29]	131 Å, 5 µm Lichrosorb, [35]	303 Å, partially sub-1 µm, silica monolith [31]
Carbon %	9.17	10.0	19.96	6.92
Hydrogen %	0.91	1.0	1.98	0.80

Table 3: Comparison of carbon and hydrogen load data for the phase of current and previous studies.

matographic performance of the resultant column [36-38] while the effect is more pronounced as far as the separation efficiency is concerned which is reported by Mazzeo., *et al.* [37]. In order to achieve the possible higher separation the standard column diameter of 4.6 mm should be used [36-38]. With decreasing the particle size lower ID column could result in higher separation efficiency. But nevertheless packing of small particles in narrow ID column is very challenging. The separation efficiency for partially sub-2 µm silica monolith particles was 164,600 plates/meter [29] and 188,300 plates/meter [30] when a column of 1 mm was used. The separation efficiency (184,000 plate/meter) for partially sub-1 µm [31] is a little lower than that of the Ref [30] where the particle size was sub-2 µm. Theoretically the separation efficiency for partially sub-1 µm should be greater than that of the partially sub-2 µm. In current study we achieved the expected separation efficiency (230,200) for partially sub-1 µm particles by the adaptation of many suitable changes throughout our current study. We incorporated many effective changes in current study such as 1) proper adjustment of column dimensions 2) catalyst assisted sophisticated modification of silica monolith particles in order to almost entirely coat surface of the silica monolith particles with polymer film 3) Suitable washing procedure in order to separate all of the polystyrene modified silica monolith particles from each other before packing 4) removal of fine particles to ensure good quality packing 5) adjusted packing protocol with respect to pressure sequence and proper vibration of the column during packing. Dealing with partially sub-1 µm silica monolith particles we had many challenges such as their modification with polystyrene, washing and packing. Modification with polystyrene causes the aggregation of major portion of polystyrene modified silica monolith particles which creates a serious problem during packing. After a series of washing steps the aggregated particles were separated which means that the particles were physically aggregated. One of the problem was the portion of fine particles present in the stationary phase of current study along with broad particle size distribution both of these problems were solved by using the sedimentation process. We removed the fine particles before modification and also right before packing by repeated sedimentation process. The sedimentation process of the stationary phase can be found in Ref. [29-31] while the sedimentation of silica monolith particles before modification was carried out by suspending the particles in a solvent mixture of 90/10 (v/v%) acetone/2-propanol in accordance with reference [34]. The suspension of silica monolith particles was stirred for 3 min, sonicated for 10 min the vial was then kept undisturbed for about 30 min. After the polystyrene bound silica monolith particles were settle down to the bottom the supernatant was removed out carefully. This process of sedimentation was repeated about five times. Instead of the tedious sieving process we adopted the easy sedimentation process in current study before modification of the silica monolith particles. The silica monolith particles obtained as a result of the sieving process still contains high population of the fine particles which deteriorate the packing quality. The % loss in bare and polystyrene modified silica monolith particles was calculated on the basis of particle size distribution data (Figure 2B in our previous study [31]). Loss of 18.8 % and 11.5 % was observed before and after modification respectively as a consequence of sedimentation treatment. Whereas this loss was only 22.4 % in the previous study [31]. Application of high pressure (2000 bar) for packing silica particles is of critical importance and is also in agreement with the data in Ref. [24,39]. Modifications in the slurry packer compatible both with high pressure (up to 1500 bar) and partially sub-1 µm particles have been carried out in current study. Mechanical vibration during column packing for different time interval is also helpful in good quality packing. For the detailed column packing procedure see Ref. [29-31]. The dimensions of slurry reservoir were adjusted according to the column dimension. The home mad connection tubing between thereservoir and column were tapered in a way to assist the smooth passage of the particles to the column. The ID of the tube connected with reservoir was similar to that of the reservoir while ID of the tube connected to the column was similar to that of the column. The N-value/meter obtained with the column of current study given in table 4 is higher than that of the core-shell particles, in addition to the high sample loading capacity of the phase of current study and low cost [40-42]. Low column back pressure of the column of current study due to its monolith like nature in comparison to that of the core shell particles [43] made it feasible to easily use these columns in conventional HPLC systems. Even the column of current study can also be used for fast HPLC analysis owing to the high permeability of the column of current study by using high pressure up to 400 bar which is under the range of conventional HPLC pump. Despite of the weakly stable pore structure and decreased mechanical stability of silica-based monolith columns [44] they are still considered as the good column category for clinical, environmental and pharmaceutical analysis regarding the separation of low molecular weight analytes. The mechanical stability of the column of current study is better than that of the typical monolith column since the silica particles are grounded and then packed in the column under high pressure which results in the packed bed with monolith like pattern having flow through channels. In contrast the first generation monolith columns are mechanically less stable since the fabricated mesh of monolith can easily breakdown by a small mechanical stress. The enhanced separation efficiency of the column of current study in comparison to the typical monolith column is due to the fact that the void spaces are far less in the packed column of small particle size. While the greater void spaces in the typical fabricated monolith column result in the increased eddy diffusion [20,45]. The repeatability data (column to column and day to day) in terms of separation efficiency and retention times obtained with column of current study are given in table 5 along with relative standard deviation. The percent relative standard deviation in separation efficiency is below 4% for column to column, and below 1% for day to day reproducibility. While the % RSD in retention time is in the range of 1 to 2 for column to column, and below 1 for day to day reproducibility. Fast HPLC analysis was also demonstrated using column of current study where all the five analytes were eluted within first 2 min using a high flow rate of 105 µL/min with good chromatographic performance. The monolith like architecture of the phase of current study allowed high influx of mobile phase during fast analysis. The separation efficiency (72700 plates/m) obtained during fast analysis for the column of current study was better than that of the previous (60,000) study [31] see table 6 and figure 5. The resolution obtained during fast analysis for current (3.12) and previous (3.28) study [31] were comparable (Table 7). The column back pressure was 397 bar during fast analysis. For the comparison of the chromatographic performance of the phase of current study with those of the previously reported and commercially available phases see Figure (4A, 4B), figure 5, and table (4, 5, 6, 7). Plate count per meter (230,200) for the phase of current study is very high. The average resolution (6.02) obtained with column of current study is better than those of the previous studies [29,30] given in Table 7, while the resolution of current study is close to that of the Ref. [31]. Similarly the average resolution (3.12) achieved during fast analysis for current study is good enough. Somewhat fast analysis is demonstrated using the column of current study shown in the form of chromatogram B in figure 4. The flow rate of the mobile phase was 105 µL/min in fast analysis where the column back pressure was 397 bar. The average number of theoretical plates as high as 72,700 plates/m have been achieved while practicing fast analysis which is higher than the average N-value obtained during the fast analysis using column of the previous study [31] figure 4B and table 6. All the analytes were eluted in the first two minutes during fast analysis in current study while in previous study all the analytes were eluted with in first 3.5 minutes.

Conclusion

Preparation of partially porous (partial sub-1 µm) silica monolith particles, their modification with polystyrene and packing in HPLC column has been demonstrated in current study. Tremendously highly separation efficiency with number of theoretical plates 230,200 plates/m has been achieved out the column packed with such particles. Particle size reduction with increased pore size, selection of proper column dimensions, catalyst assisted ligand binding, sedimentation protocol and the modified packing procedure has made it possible to achieve very high separation efficiency for the column of current study which is greater than that of any reported particle packed column up till now. The column of current study was capable of fast analysis where all the five analytes were eluted within first two minutes.

	Specifications of stationary phases and columns							
Analytes	**Partially sub-1µm silica monolith particles**				**Partially sub-2 µm**		**3 µm C18**	
	Polystyrene bound phase, Current study a 1.8 mm ID X 300 mm		**C18-modified phase [31] b 1.8 mm ID X 150 mm**		**Polystyrene bound phase [29] c 1 mm ID X 300 mm**		**Commercial Alltima d 1.0 mm×150 mm**	
	Plate count	**HETP**	**Plate count**	**HETP**	**Plate count**	**HETP**	**Plate count**	**HETP**
1	242,300± 1300	4.13	146666 ± 400	6.8	179000±1500	5.5	78000 ± 250	12.7
2	232,400 ± 950	4.30	193333 ± 940	5.2	174333 ± 900	5.7	106666 ± 220	9.38
3	228,000± 1130	4.39	194666 ± 1200	5.1	172000±1100	5.8	106666 ± 200	9.4
4	244,100± 1080	4.10	192666 ± 1260	5.2	144666±1500	6.9	91333 ± 380	10.9
5	204,400 ± 850	4.89	192000 ± 1020	5.2	153333±1400	6.5	88000 ± 410	11.3
Average	230,200	4.36	183932	5.4	164666	6.07	94133	10.6

Table 4: Comparison of plate count/m and plate height (µm) of the column of current study with those of the previous studies.

1) Phenol 2) Acetophenone 3) 4-Methyl-2-nitor-anailine 4) Benzene and 5) Toluene.

The mobile phase was Acetonitrile/water (v/v), 0.1% TFA that is 65/35 for a, 60/40 for c, and 70/30 for b, d. Flow rate (µL/min): 28 for a 25 for b 15 for c and 7 for d. Detection wavelength: 214 nm for all the studies.

Analytes	**Column to column reproducibility**				**Day to day reproducibility**			
	Average N-value	**% RSD**	**Average Retention time**	**% RSD**	**Average N-value**	**% RSD**	**Average Retention time**	**% RSD**
Phenol	240700	2.9%	11.73%	1.4%	241700	0.7%	11.71%	0.5%
Acetophenone	233000	3.5%	12.87%	1.9%	231300	0.8%	12.89%	0.7%
4-Methyl-2-nitroaniline	228300	3.5%	13.17%	1.3%	229600	0.6%	13.14%	0.6%
Benzene	244100	3.8%	14.11%	2.1%	245200	0.9%	14.09%	1.0%
Toluene	205700	3.3%	15.01%	1.8%	204700	1.1%	15.05%	0.8%

Table 5: Plate count/m and retention time (min) reproducibility data for the column of current study.

To check the day to day reproducibility in plate count and retention time a single column packed with the phase of current study was analyzed on three different days in one week while three columns packed with the phase of current study were checked for column to column reproducibility. The average N-value and retention time of three data for column to column and day to day reproducibility are given in the table.

Analytes	**Phenol**	**Acetophenone**	**4-Methyl-2-nitroaniline**	**Benzene**	**Toluene**	**Average**
Current study	75700 ± 90	59200 ± 110	62800 ± 130	78500 ± 100	87400 ± 100	72700
Previous study [31]	67333 ± 15	61000 ± 120	65333 ± 40	54000 ± 80	52000 ± 110	60000

Table 6: Comparison of N-values obtained during fast analysis for the column of current study a, with those of the previous study [b].

Fast analysis was carried out using high flow rate (105 µL/min) with a detection wavelength of 214 nm for both a, b. All the five analytes were eluted within first two minutes for a.

Mobile phase; 65:35 v/v acetonitrile/water, 0.1% TFA. Flow rate; 105 µL/min.

Mobile phase; 70:30 v/v acetonitrile/water with 0.1% TFA. Flow rate; 100 µL/min.

Stationary phase	Solute pair [a]				
	1-2	2-3	3-4	4-5	Average
Polystyrene bound phase of Current study [b]	8.09	3.50	6.31	6.21	6.02
Fast analysis associated with current study [b1]	4.03	1.01	3.91	3.53	3.12
C18-bound phase [31] [c]	7.15	2.70	7.05	7.30	6.05
Fast analysis associated with previous study [c1]	3.93	1.52	3.98	3.71	3.28
Polystyrene-bound phase [30] [d]	8.15	3.82	5.25	6.31	5.88
Polystyrene-bound phase [29] [e]	5.15	1.43	3.76	3.35	3.42
Alltima commercial C18 (3µm) [f]	7.37	2.73	6.93	7.08	6.02

Table 7: Comparison of resolution (under optimized elution conditions) between current and previous studies and resolution comparison obtained during fast analysis between current and previous study [31].

a) Solutes: 1, phenol; 2, acetophenone; 3, 4-methyl-2-nitronaniline; 4, benzene; 5, toluene.

The mobile phase was acetonitrile/waster (v/v %), 0.1% TFA in all the studies having different composition of water and acetonitrile.

For b and b1) column; 1.8 mm ID × 300 mm long. Mobile phase; 65/35 where the flow rate was 28 µL/min for b and 105 µL/min for b1.

For c, c1, and f) column; 1.0 mm ID × 150 mm long. Mobile phase; 70:30 where the flow rate was 25 µL/min for c and g, while it was 100 µL/min for c1.

For d and e) column; 1.0 mm ID × 300 mm long column. Mobile phase; 60:40 at a flow rate of 15 µL/min.

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Conflict of Interest

We (Dr. Faiz Ali, Professor Won Jo Cheong Dr. Najeeb-Ur-Rehman and Aamra Rafique Malik the authors of the manuscript) declare that we don't have any financial/commercial conflicts of interest on this manuscript.

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