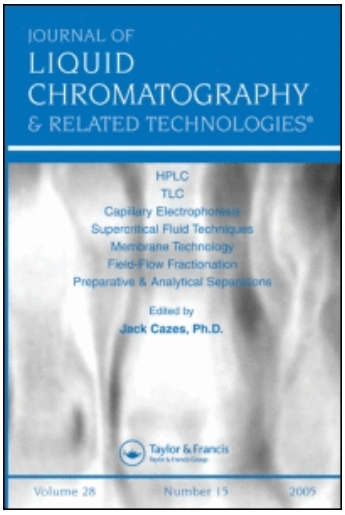

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Rapid Method for Analysis of Aspirin-Butalbital in Serum Utilizing a Monolithic C_{18} Column

C. Pistos[a]; J. T. Stewart[a]
[a] Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, Georgia, USA

Online publication date: 28 July 2003

To cite this Article Pistos, C. and Stewart, J. T.(2003) 'Rapid Method for Analysis of Aspirin-Butalbital in Serum Utilizing a Monolithic C_{18} Column', Journal of Liquid Chromatography & Related Technologies, 26: 15, 2567 — 2579
To link to this Article: DOI: 10.1081/JLC-120023801
URL: http://dx.doi.org/10.1081/JLC-120023801

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 26, No. 15, pp. 2567–2579, 2003

Rapid Method for Analysis of Aspirin–Butalbital in Serum Utilizing a Monolithic C_{18} Column

C. Pistos and J. T. Stewart*

Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, Georgia, USA

ABSTRACT

A fast and sensitive high performance liquid chromatography (HPLC) assay was developed for the simultaneous determination of aspirin–butalbital in human serum. Serum samples were treated with a solid phase extraction procedure. The analytes were separated using a mobile phase of 90 : 10 (v/v) 0.1 M aqueous potassium phosphate monobasic (pH 4)-acetonitrile on a monolithic C_{18} column with UV detection at 220 nm. Benzoic acid was used as the internal standard (IS). The method was validated over the range of 0.5–100 $\mu g\,mL^{-1}$ for each drug. The method proved to be accurate (percent bias for all calibration samples varied from −13 to 6.6%) and precise (range from 0.1% to 10%). The mean percent absolute recoveries were 104 ± 6.3 for aspirin, 92.6 ± 5.5 for butalbital and 106 ± 6.9 for the internal standard. The assay should be

*Correspondence: J. T. Stewart, Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA 30602-2352, USA; E-mail: jstewart@rx.uga.edu.

DOI: 10.1081/JLC-120023801
1082-6076 (Print); 1520-572X (Online)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

applicable for use in pharmacokinetic studies and routine serum monitoring of these drugs.

Key Words: Monolithic C_{18} column; Aspirin–butalbital mixture; Human serum; HPLC.

INTRODUCTION

The ever increasing need for speed and efficient use of time in pharmaceutical analysis places a demand for the development of faster and higher throughput procedures. The recent introduction of commercially available silica-based monolithic C_{18} high performance liquid chromatography (HPLC) columns presents an alternative to conventional packed silica columns. Produced via sol–gel chemistry and using technology developed by Nakanishi and Soga[1] these porous monoliths are designed such that a tightly controlled bimodal pore size distribution is obtained. Bulk mobile phase flow occurs through macropores, nominally 2 μm in diameter. The structure and geometry of the macropores impart to the monolith a lower flow resistance relative to a packed bed column. Mesopores having a nominal diameter of 130 Å provide the surface area needed for stationary phase coverage and analyte partitioning. The ability during synthesis to control the size of the macropores relative to the silica skeleton is the key to the monolithic column. The enhanced permeability of these columns allows separations to be conducted at high flow rates ($>5\,mL\,min^{-1}$) without excessive backpressure. In addition, the monoliths are claimed to provide reduced band broadening, arising from stagnant mobile phase mass transfer, and thereby minimizing efficiency loss at high linear velocities. The main advantage of monolithic columns is that analysis time can be decreased without compromising resolution.[2]

Although the column technology in HPLC has reached a high standard of quality and high reproducibility, attempts have been undertaken to search for a novel column design based on monolithic supports rather than microparticular packings.[3–5] The advancements achieved in the sol–gel chemistry of silica[6] provide the material basis to design composite supports of silica and polymers with a coherent binary phase system.[7] Such composites have been converted into porous bodies after calcination, i.e., removal of the polymeric constituent. The porous silica bodies shaped as rods[8] exhibit a bimodal and highly connected pore system composed of flow-through pores of 1–2 μm in width and mesopores with adjustable average pore diameter.[9]

Besides its interesting hydrodynamic characteristic, the preparation of the monolithic column has another advantage. In contrast to particle preparation, where common particle size classification is required after polymerization is

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

completed, monoliths are prepared via a bulk polymerization procedure and their structure is defined by monomer composition and polymerization temperature without further processing.[10,11]

Several monolithic supports were described in the literature in the late 1980s and early 1990s. They were synthesized from different chemical compounds to form acrylamide,[12] silica,[13] styrene,[14] and methacrylate[15] monoliths.

A limited number of bioanalytical applications of monolithic columns have appeared in the literature.[16,17] Each of the published applications has used tandem mass spectrometry (MS) as the chromatographic detection method. Tandem MS detection requires that the monolithic column effluent flow be split prior to the MS interface, as currently available MS interfaces cannot tolerate flow-rates in excess of 2 mL min^{-1}. Flow splitting potentially limits assay sensitivity as a portion of the injected sample is diverted to waste.[1]

Srinivasan and Bartlett and our laboratory have previously reported analytical methods for butalbital in serum[18] and aspirin–butalbital mixtures in dosage forms[19] using capillary electrophoresis and an HPLC assay for aspirin–butalbital tablets.[20] No articles have been reported in the literature for the concurrent determination of aspirin–butalbital in serum. The major problem in developing such a determination is the difference in lipophilicity between the two analytes. Furthermore, the sensitivity of butalbital is very low, and does not allow for optimization of the separation and sensitivity of the method at wavelengths greater than 220 nm. Using such a method, in addition to the interferences by endogenous serum peaks, assay time would probably be long.

This paper describes the development and validation of an HPLC assay using a monolithic C_{18} column that is both rapid and sensitive for determining aspirin–butalbital in human serum. Therapeutic levels of these drugs in serum will likely be in the 10–65 μg mL^{-1} range, respectively. For sample pre-treatment, the method utilizes solid phase extraction and elution was performed isocratically with UV detection at 220 nm.

EXPERIMENTAL

Chemicals and Supplies

Aspirin and butalbital were purchased from Sigma Chemical Co. (St. Louis, MO). Benzoic acid was obtained as a USP reference standard (United States Pharmacopeia, Rockville, MD). Acetonitrile, methanol, and potassium phosphate monobasic were obtained from Fisher Scientific

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

(Fair Lawn, NJ), and phosphoric acid was purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Water was purified by a cartridge system obtained from Continental Water System (Roswell, GA). Drug free human serum was secured from Bioreclamation Inc. (Hicksville, NY).

Instrumentation

The HPLC system consisted of a Model 110 A pump (Beckman, Fullerton, CA), a Model 759 A variable wavelength UV/VIS detector (Applied Biosystems, Foster City, CA), and a Model 3394 A integrator (Hewlett Packard, Palo Alto, CA). A Valco Model C6W injection system (Valco Instrument Co, Houston, TX) equipped with a 20-μL loop was used for injection. The analytical column used was a Chromolith performance RP-18e (10 cm × 4.6 mm ID) and was purchased from Merck KgaA, Darmstadt, Germany via Phenomenex (Torrance, CA). The monolithic C_{18} column was protected with a classic C_{18} guard column (7.5 × 4.6 mm), which was supplied from Alltech Associates, Inc. (Deerfield, IL).

Chromatographic Conditions

The mobile phase consisted of 90 : 10 (v/v) 0.1 M aqueous potassium phosphate monobasic (pH 4)-acetonitrile. The mobile phase was filtered through a 0.22 μm nylon-66 filter (MSI, Westborough, MA) and degassed before use. The HPLC pump flow rate was 9 mL min^{-1} and all analyses were conducted at ambient temperature (23°C ± 1°C). The UV detector was set at 220 nm.

Preparation of Combined Standard Solution

A combined stock solution of aspirin, butalbital, and benzoic acid (IS) was prepared by dissolving appropriate amounts of each drug in acetonitrile to obtain final drug concentrations of 500 μg mL^{-1}. The stock solution was stored at 4°C.

Sample Preparation Procedure

Calibration standards and quality control samples were prepared by adding appropriate aliquots of the combined drug stock solution into 1 mL of filtered drug free human serum containing 50 μL of concentrated phosphoric acid. Each sample was then vortexed for 30 s. Solid phase extraction

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

cartridges (Varian Inc., bonded phase Nexus 10 cc/30 mg) were placed on a vacuum elution apparatus (Analytical International, Harbor City, CA) and rinsed with 1 mL of methanol, followed by 1 mL of purified water acidified to pH 1.5. Care was taken that the cartridges did not run dry. One milliliter of each standard or QC sample was then transferred to the SPE cartridges. Vacuum was applied to obtain a flow through the cartridges of 1–2 mL min^{-1} followed by vacuum suction for 1 min. The analytes were eluted from the cartridges using 1 mL of methanol basified to pH 12.5 with concentrated sodium hydroxide, followed by vacuum suction for 1 min. Extracts were then collected, vortex mixed for a few seconds, and 20 μL was then injected into the liquid chromatograph.

Specificity

The specificity of the assay was checked by analyzing three independent blank human serum samples. The chromatograms of these blank serum samples were then compared with chromatograms obtained by analyzing human serum samples spiked with the analytes.

Linearity

Calibration plots for the analytes in serum were prepared by diluting stock solutions with filtered serum containing phosphoric acid to yield concentrations in the 0.5–100 μL mL^{-1} range (0.5, 0.75, 1, 2.5, 6, 10, 12.5, 15, 20, 40, 60, 80, and 100 μg mL^{-1}). Calibration standards at each concentration were subjected to SPE and analyzed in duplicate. Calibration curves were constructed using ratios of the observed analyte peak height to IS vs. nominal concentration of analyte. Linear regression analysis of the data gave slope, intercept, and correlation coefficient data. From this data, a first order polynomial model was selected for each analyte.

Precision and Accuracy

The accuracy and precision of the assay in serum were determined by assaying six quality control samples of aspirin and five of butalbital in triplicate over a period of three days. The concentrations represented the entire range of the calibration curves. The lowest level was at the expected limit of quantification (LOQ) for each analyte (1.5 μg mL^{-1}). The highest level was at 80% of the upper boundary of the calibration curves (80 μg mL^{-1}). The nine measured concentrations per concentration level (triplicates for three

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

runs) were subjected to analysis of variance (ANOVA) to estimate precision. Percent accuracy was determined (using the data from the precision assessment) as the closeness of spiked samples to the nominal value of in-house standards. Precision was reported as percent relative standard deviation (%RSD).

Limit of Detection and Limit of Quantification

The limit of detection (LOD) was defined as the concentration that yields a signal-to-noise ratio of 3. The LOQ was calculated to be the concentration that yields a signal-to-noise ratio of 10.

Recovery

The absolute recoveries of aspirin, butalbital, and benzoic acid from human serum were assessed at three concentrations (1.5, 20, and 60 $\mu g\,mL^{-1}$). For each level, three samples were extracted and analyzed in triplicate. Three replicates of each concentration prepared in the eluent were directly injected. The absolute recovery for each analyte, at each concentration, was computed using the following equation: absolute recovery = (mean peak height in extract)/(mean peak height in eluent) $\times$ 100.

RESULTS AND DISCUSSION

The chemical structures for aspirin, butalbital, and the IS benzoic acid are shown in Fig. 1 (A, B, and C). The primary advantage in developing this monolithic method is a chromatography run time of less than 4 min using a simple SPE method. The large difference in lipophilicity between aspirin and butalbital posed an initial challenge in analytical development. The more hydrophilic aspirin and benzoic acid tended to elute with endogenous substances in the serum extract, whereas butalbital eluted much later in the run. A chromolith RP-18e column was selected for the separation of the analytes and IS utilizing an isocratic run. The mobile phase was 90 : 10 (v/v) 0.1 M aqueous potassium phosphate monobasic (pH 4)-acetonitrile. The flow rate of 9 $mL\,min^{-1}$ did not yield a back pressure higher than 2600 psi and allowed a very fast separation (<4 min). The detector was set at 220 nm in order to obtain an optimized separation in terms of the lipophilicity and sensitivity of the analytes. Phosphoric acid was added to adjust the mobile phase pH. These conditions gave good selectivity and sensitivity for both drugs in an assay time less than 4 min.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

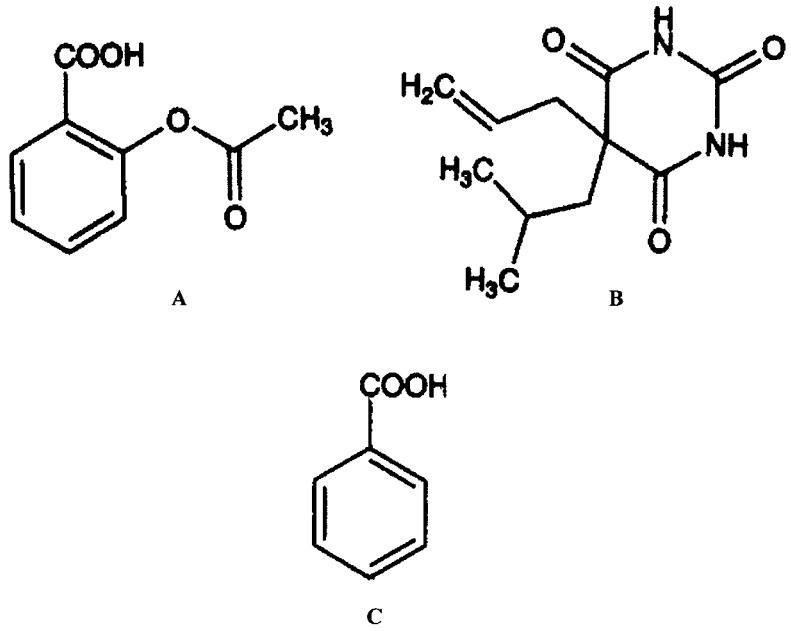

Figure 1. Chemical structures of A, aspirin; B, butalbital; and C, benzoic acid.

The primary objective in the development of the SPE extraction method was to minimize interfering endogenous sample components, while at the same time providing maximum recoveries of the analytes. During development of the solid phase extraction method, a series of different extraction cartridges were investigated, such as C_{18}, C_8, Nexus, and SAX cartridges. The Nexus cartridge was found to give the best recoveries, while at the same time removing endogenous serum interferences. One milliliter of acidified water (pH 1.5) was used to help retain the analytes before loading of the spiked serum samples. Basified methanol (pH 12.5) was strong enough to elute the analytes and IS.

Specificity

During our preliminary experiments, several combinations of the mobile phase composition and pH were investigated in order to obtain the optimum separation. Thus, phosphate buffer concentrations at 0.025, 0.05, 0.1, and 0.15 M, mobile phase pH at 3, 4, 5.5, and 7.1, and acetonitrile percentages in

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

the mobile phase at 5%, 7.5%, 10%, 12.5%, and 15% were examined. In the first case, it was observed that molarity of the phosphate buffer in the mobile phase affected retention time of the analytes. A 0.1 M buffer concentration gave the best separation in the shortest elution time, since at a lower concentration of the buffer, the aspirin peak was overlapped or was interfered with by the serum peaks (Fig. 2A). In the second case, the mobile phase pH affected retention time and separation in a similar fashion. The result was an overlapping of aspirin and serum protein peaks at higher than pH 4 values, which consequently led to a separation failure (Fig. 2B). The retention times for the analytes increased as the percentage of acetonitrile in the mobile phase decreased (Fig. 2C). The result was more significant for the lipophilic molecule butalbital.

The best separation of aspirin and butalbital on the monolithic C_{18} column was achieved using a mobile phase of 90 : 10 (v/v) 0.1 M aqueous potassium phosphate buffer (pH 4)-acetonitrile with retention times of 0.73 min for aspirin and 3.69 min for butalbital. The IS (benzoic acid) was well resolved from aspirin with a retention time of 1.07 min. No interferences were observed in drug free human serum samples. Figure 3A, B shows chromatograms of a blank sample and a spiked sample, respectively.

Linearity

The calibration curves showed good linearity in the range of 0.5–100 $\mu g\,mL^{-1}$ for each analyte. The correlation coefficients (r) of calibration curves of each drug were higher than 0.996 as determined by least square analysis.

Precision and Accuracy

A summary of the accuracy and precision results is given in Table 1. The method proved to be accurate (percent bias for all calibration samples varied from −13% to 6.6%) and precise (range from 0.1% to 10%). The acceptance criteria %RSDs of <15% and an accuracy (between 85% and 115%) were met in all cases.

Limit of Detection and Limit of Quantification

The LOD was 0.5 $\mu g\,mL^{-1}$ and the LOQ was 1.5 $\mu g\,mL^{-1}$ for each drug. Limit of detection and LOQ data are shown in Table 2.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

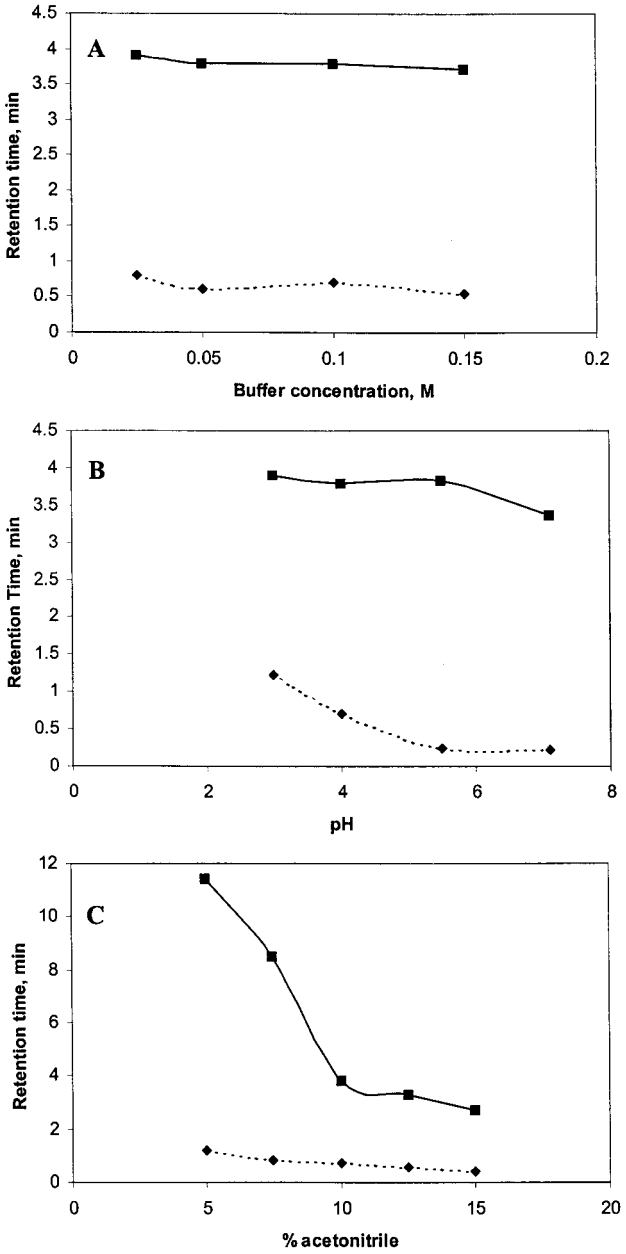

Figure 2. Effect of A, buffer concentration; B, pH; and C, percentage of acetonitrile on the retention time of aspirin (···◆···) and butalbital (—■—).

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

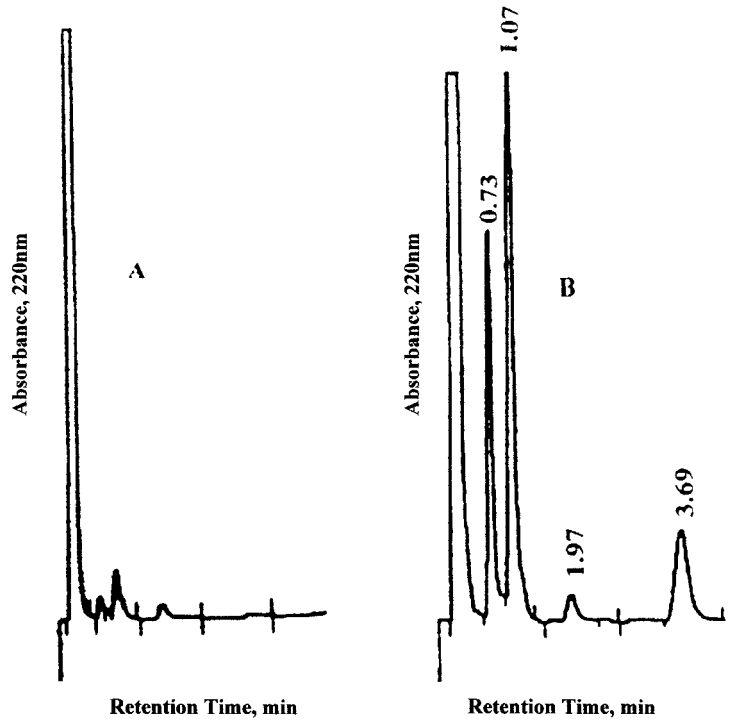

Figure 3. Typical chromatograms of A, blank serum and B, spiked serum containing 12.5 μg mL^{-1} each of aspirin (0.73 min), butalbital (3.69 min), and benzoic acid (1.07 min). HPLC conditions: monolithic C_{18} column; mobile phase of 90 : 10 (v/v) 0.1 M aqueous potassium phosphate monobasic (pH 4)-acetonitrile; flow rate of 9 mL min^{-1}; UV detection at 220 nm, injection volume of 20 μL, and ambient temperature (23°C).

Recovery

The mean absolute percent recoveries were 104 ± 6.3 for aspirin, 92.6 ± 5.5 for butalbital, and 106 ± 6.9 for benzoic acid (internal standard).

CONCLUSION

An HPLC method using a monolithic C_{18} column was developed and validated for the determination of aspirin–butalbital in human serum. The method combines a solid phase extraction procedure with a fast and sensitive

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Table 1. Intra- and inter-day accuracy and precision assay for aspirin–butalbital in serum.

	Concentration ($\mu g\,mL^{-1}$)			
	Added	Found	Error (%)	RSD (%)
Intra-day				
Aspirin ($n=6$)	1.5	1.6 ± 0.16	6.6	10
	4.5	4.8 ± 0.02	6.6	0.4
	20	20.8 ± 0.05	4	0.2
	40	41.5 ± 1.2	3.8	3
	60	62.3 ± 2.2	3.8	3.5
	80	84 ± 3.3	5	4
Butalbital ($n=5$)	1.5	1.36 ± 0.03	−9.3	2.2
	3	2.82 ± 0.1	−6	3.5
	7	6.78 ± 0.1	−3.1	1.5
	10	8.9 ± 0.7	−11	7.9
	13	11.3 ± 1	−13	8.8
Inter-day				
Aspirin ($n=18$)	1.5	1.6 ± 0.14	6.6	8.7
	4.5	4.7 ± 0.01	4.4	0.2
	20	21 ± 0.03	5	0.1
	40	41.7 ± 0.9	4.2	2.2
	60	62.5 ± 2.1	4.2	3.4
	80	83.7 ± 3.6	4.6	4.3
Butalbital ($n=15$)	1.5	1.36 ± 0.02	−9.3	1.5
	3	2.85 ± 0.1	−5	3.5
	7	6.8 ± 0.2	−2.8	2.9
	10	8.7 ± 0.8	−13	9.2
	13	11.6 ± 1.1	−10.8	9.4

Table 2. Range of calibration curves, LOD, and LOQ ($\mu g\,mL^{-1}$) of aspirin and butalbital in spiked human serum.

Drug	Range of calibration curves	Limit of detection[a]	Limit of quantitation[b]
Aspirin	0.5–100	0.5	1.5
Butalbital	0.5–100	0.5	1.5

[a]$S/N = 3$.
[b]$S/N = 10$.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

isocratic reversed phase HPLC analysis with UV detection at 220 nm. The method should be suitable for monitoring drug concentrations in human serum and for pharmacokinetic studies.

REFERENCES

1. Nakanishi, K.; Soga, N. Phase separation in silica sol–gel system containing polyacrylic acid 1. Gel formation behavior and effect of solvent composition. J. Non-Cryst. Solids **1992**, *139*, 1–13.
2. Vallano, P.T.; Mazenko, R.S.; Woolf, E.J.; Matuszewski, B.K. Monolithic silica liquid chromatography columns for the determination of cyclooxygenase II inhibitors in human plasma. J. Chromatogr. B **2002**, *779*, 249–257.
3. Svec, F.; Frechet, J.M.J. Continuous rods of macroporous polymer as high performance liquid chromatography separation media. Anal. Chem. **1992**, *64*, 820–822.
4. Wang, Q.C.; Švec, F.; Frechet, J.M.J. Reversed-phase chromatography of small molecules and peptides on a continuous rod of macroporous poly (styrene-co-divinylbenzene). J. Chromatogr. A **1994**, *669*, 230–235.
5. Hjertén, S.; Li, Y.-M.; Liao, J.-L.; Mohammad, J.; Nakazato, K.; Pettersson, G. Continuous beds-high-resolving, cost-effective chromatographic matrices. Nature **1992**, *356*, 810–811.
6. Brinker, C.J.; Scherer, G.W.; *Sol-Gel Science: The Physics and Chemistry of Sol-Gel Processing*; Academic Press: New York, N.Y., 1990.
7. Nakanishi, K. Pore structure control of silica gels based on phase separation. J. Porous Mat. **1997**, *4*, 67–112.
8. Cabrera, K.; Weiland, G.; Lubda, D.; Nakanishi, K.; Soga, N.; Minakuchi, H.; Unger, K.K.; SilicaROD—a new challenge in fast high-performance liquid chromatography separations. Trends Anal. Chem. **1998**, *17*, 50–53.
9. Bidlingmaier, B.; Unger, K.K.; Von Doehren, N. Comparative study on the column performance of microparticulate 5 μm C_{18}-bonded and monolithic C_{18}-bonded reversed-phase columns in high-performance liquid chromatography. J. Chromatogr. A **1999**, *832*, 11–16.
10. Podgorning, A.; Barut, M.; Štrancar, A.; Josic, D.; Koloini, T. Construction of large-volume monolithic columns. Anal. Chem. **2000**, *72*, 5639–5699.
11. Švec, F.; Fréchet, J.M. Molded rigid monolithic porous polymers: an inexpensive, efficient, and versatile alternative to porous beads for the design of materials for numerous applications. Ind. Eng. Chem. Res. **1999**, *38*, 34–48.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

12. Hjertén, S.; Liao, J.L.; Zhang, R.J. High-performance liquid chromatography on continuous polymer beds. J. Chromatogr. A **1989**, *473*, 273–275.
13. Nakanishi, B.K.; Soga, N. Phase separation in gelling silica organic polymer solutions: systems containing poly (sodium styrenesulfonate). J. Am. Ceram. Soc. **1991**, *74*, 2518–2530.
14. Wang, Q.C.; Švec, F.; Frechet, J.M.J. Macroporous polymeric stationary-phase rod as continuous separations medium for reversed-phase chromatography. Anal. Chem. **1993**, *65* (17), 2243–2248.
15. Tennikova, T.B.; Belenkkii, B.C.; Švec, F. High-performance membrane chromatography. A novel method of protein separation. J. Liq. Chromatogr. **1990**, *13*, 63–70.
16. Dear, G.; Plumb, R.; Mallett, D. Use of monolithic silica columns to increase analytical throughput for metabolite identification by liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. **2001**, *15*, 152–158.
17. Plumb, R.; Dear, G.; Mallett, D.; Ayrton, J. Direct analysis of pharmaceutical compounds in human plasma with chromatographic resolution using an alkyl-bonded silica rod column. Rapid Commun. Mass Spectrom. **2001**, *15*, 986–993.
18. Srinivasan, K.; Bartlett, M.G. Capillary electrophoresis determination of butalbital from serum using solid phase extraction (SPE) and ultraviolet detection. J. Liq. Chromatogr. Relat. Technol. **2000**, *23* (15), 2361–2371.
19. Haque, A.; Stewart, J.T. Simultaneous determination of codeine, caffeine, butalbital and aspirin by free solution capillary electrophoresis. J. Liq. Chromatogr. Relat. Technol. **1999**, *22* (8), 1193–1204.
20. Xu, X.; Stewart, J.T. HPLC methods for aspirin-caffeine-butalbital and acetaminophen-caffeine-butalbital mixtures in tablet dosage forms using non-porous octadecylsilane columns. J. Liq. Chromatogr. Relat. Technol. **2000**, *23* (5), 769–779.

Received February 24, 2003
Accepted March 20, 2003
Manuscript 6095

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016