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Assay for the simultaneous determination of acetaminophen–caffeine–butalbital in human serum using a monolithic column

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Abstract

A fast and sensitive high performance liquid chromatography (HPLC) assay was developed on a C18 monolithic column for the simultaneous determination of acetaminophen–caffeine–butalbital in human serum. Serum samples were treated with a solid phase extraction procedure. The analytes were separated using a mobile phase of 95:5 (v/v) 0.1 M potassium phosphate monobasic (pH 2.41)–acetonitrile on the C18 monolithic column with detection at 220 nm. Benzoic acid was used as the internal standard (IS). The method was validated over the range of 1.25–100 μg/ml for each drug and found to be linear ($r > 0.995$, $n = 12$) with RSD less than 8.3%. The method proved to be accurate (percent bias for all calibration samples varied from −14.6 to −1.3%) and precise (ranged from 2.9 to 13.4%). The mean percent absolute recoveries from serum were 89.7 ± 3.6 for acetaminophen, 95.5 ± 4.5 for caffeine, 99 ± 5.2 for butalbital and 83.4 ± 3.9% for the internal standard.

Keywords: Monolithic column; Acetaminophen–caffeine–butalbital; Serum; HPLC

1. Introduction

An ever-increasing need exists within the pharmaceutical industry to shorten the drug development timeline. Maximizing the throughput of assays that support clinical studies is, therefore, a key concern in a bioanalytical laboratory. For HPLC-based assays, the process of reducing analysis time while adequately resolving analytes from endogenous components and metabolites is often accomplished with short columns ($L \leq 50$ mm) packed with small particles (d_p = 3 μm). The theoretical advantages of small packing particles include higher optimum linear velocities as well as shallower slopes in the high velocity region of plate height versus linear velocity curves. In principle, high speed and high resolution separations can be obtained by operating small particle packed columns at high flow rates. Unfortunately, the high back pressure associated with these columns effectively limits their operation to mobile phase flow rates of less than about 2 ml/min [1].

Monolithic columns have generated interest as an alternative to particulate columns [2–6]. Monolithic columns possess a unique biporous structure. The main difference in comparison to conventional particle beds is in their structure. Conventional particle-based supports consist of few micrometer-sized porous particles. Because the pores within the particles are close to each other, the liquid inside them is stagnant. Therefore, the molecules to be separated are transported to the active sites inside the close pores and back to the mobile phase mainly by diffusion. Since diffusion itself is a rather slow process, especially in the case of large molecules with a low mobility, it determines the overall separation time. Furthermore, the separation efficiency depends on the residence time of the sample inside the stationary phase and, therefore, on the linear velocity of the mobile phase through the separation medium [7]. Monoliths, on the other hand, consist of

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a single piece of porous material. The mesopores located on the silica skeleton (13 nm) are highly interconnected, forming a network of channels and providing the large surface area needed to achieve sufficient capacity. Meanwhile, the larger through pores (2 μm) reduce flow resistance, allowing the use of high flow rates at considerably reduced backpressure. Since the flow of the liquid within the channels is driven by the pressure difference, the molecules to be separated are transported to the active sites located on the surface of the channels by convection, increasing their mobility by several orders of magnitude. Thus, monolithic columns of conventional lengths (<5 cm) can be used under high flow rates to achieve sufficient resolving power. The dependency of separation efficiency on flow rate is extremely small, therefore, high separation efficiency can be maintained at significantly increased flow rates [5]. While these columns have been used to analyze metabolites [6] and natural products [8], the capability of this type of column for routine high-throughput bioanalysis in a drug discovery environment has not been demonstrated [9].

There are several different monolithic supports described in the literature introduced in the late 1980s or early 1990s. They are basically synthesized from different chemical compounds to form acrylamide [10], silica [11], styrene [12] and methacrylate [13] monoliths. In contrast to particle preparation, where commonly particle size classification is required after polymerization is completed, monoliths are prepared with a bulk polymerization and their structure is defined already by monomer composition and polymerization temperature without further processing [14,15].

A limited number of bioanalytical applications of the monolithic column has appeared in the literature [6,16,17]. Our laboratory was particularly interested in the application of the C18 monolithic column to the concurrent assay of an acetaminophen–caffeine–butalbital mixture in serum that would be both rapid and sensitive. Srinivasan and Bartlett have previously reported analytical methods for butalbital in serum [18] using capillary electrophoresis. Our laboratory has reported methods for acetaminophen–caffeine–butalbital mixtures in tablet dosage forms by micellar electrokinetic chromatography [19] and HPLC assays for aspirin–caffeine–butalbital and acetaminophen–caffeine–butalbital mixtures in tablet dosage forms [20]. No articles have been reported in the literature for the concurrent determination of acetaminophen–caffeine–butalbital in serum. The major problem in developing such an assay for the three analytes in serum is the difference in lipophilicity. In addition, the sensitivity of butalbital is very low and this does not allow for better optimization of the drug at wavelengths higher than 220 nm. Furthermore, there is possible interference by the endogenous serum peaks and HPLC retention times can be long.

This paper describes the development and validation of an assay that is both rapid and sensitive for determining acetaminophen–caffeine–butalbital in human serum. After oral administration of the drug mixture, therapeutic levels of the drugs in serum are in the 8–65 μg/ml range. The method utilizes solid phase extraction for sample pretreatment and elution is performed isocratically at 220 nm detection.

2. Experimental

2.1. 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 column was protected with a C18 guard column (7.5 mm × 4.6 mm) which was supplied from Altech Associates, Inc. (Deerfield, IL).

2.2. Reagents and chemicals

Acetaminophen, caffeine and butalbital were purchased from Sigma Chemical Co., St. Louis, MO. Benzoic acid was a USP reference standard (United States Pharmacopeia, Rockville, MD). Acetonitrile, methanol and potassium phosphate monobasic were obtained from Fisher Scientific (Fair Lawn, NJ) and phosphoric acid by J.T. Baker Chemical Co., Phillipsburg, N.J. Water was purified by a cartridge system (Continental Water System, Roswell, GA). Drug-free human serum was obtained from Bioreclamation Inc. (Hicksville, NY).

2.3. Chromatographic conditions

The mobile phase consisted of 95:5 (v/v) 0.1 M potassium phosphate monobasic (pH 2.41)–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 and all analyses were conducted at ambient temperature (23 ± 1 °C). The UV detector was operated at 220 nm.

2.4. Sample preparation

A combined stock solution of acetaminophen, caffeine, butalbital and benzoic acid was prepared by dissolving appropriate amounts of each drug in acetonitrile to obtain final drug concentrations of 500 μg/ml. The stock solution was stored at 4 °C.

The primary objectives in the development of the extraction method were to minimize interfering endogenous sample components, while at the same time providing high 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 highest recoveries, while at the same time removing endogenous interferences.

Calibration standards and quality control samples were prepared by adding appropriate aliquots of the stock solution into 1 ml of filtered drug-free serum containing 50 μl of concentrated phosphoric acid. Each of the samples was then vortexed for 30 s. Solid phase extraction cartridges (Varian Inc., Bonded phase Nexus 10 cm^3/30 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 sample was transferred to the SPE cartridges. Vacuum was then applied to obtain a flow through the cartridges of 1–2 ml/min followed by vacuum suction for 1 min. The analytes were then 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 and 20 μl was then injected into the liquid chromatograph.

3. Results and discussion

The chemical structures for acetaminophen, caffeine, butalbital and the internal standard benzoic acid are shown in Fig. 1. The advantage in developing this method was a run time of less than 10 min, using a simple SPE method. The large difference in lipophilicity between acetaminophen–caffeine–butalbital posed a challenge in the development of the separation. The more hydrophilic acetaminophen, caffeine and benzoic acid tended to elute with endogenous substances in the serum extract, whereas butalbital tended to elute much later in the run. The chromolith RP-18e column was selected which yielded the advantage to optimize the separation of such drugs by utilizing an isocratic run.

During our preliminary experiments, we tried several combinations of the mobile phase composition and pH in order to obtain the optimum separation. Thus, we examined buffer concentrations at 0.025, 0.05, 0.1 and 0.15 M potassium phosphate monobasic, mobile phase pHs at 2.4, 2.5, 5.25 and 7.1, and acetonitrile percentages in the mobile phase at 2.5, 5, 6, 7, 10 and 12.5%. In the first case, it was observed that potassium phosphate monobasic concentration in the mobile phase affected retention time of the analytes. It was found that a 0.1 M buffer concentration gave the best separation in the shortest elution time, since at lower concentration of the buffer, the acetaminophen peak overlapped or was interfered with by the serum peaks (Fig. 2A). In the second case, pH affected retention time and separation in a similar fashion. The result was an overlapping of acetaminophen and serum

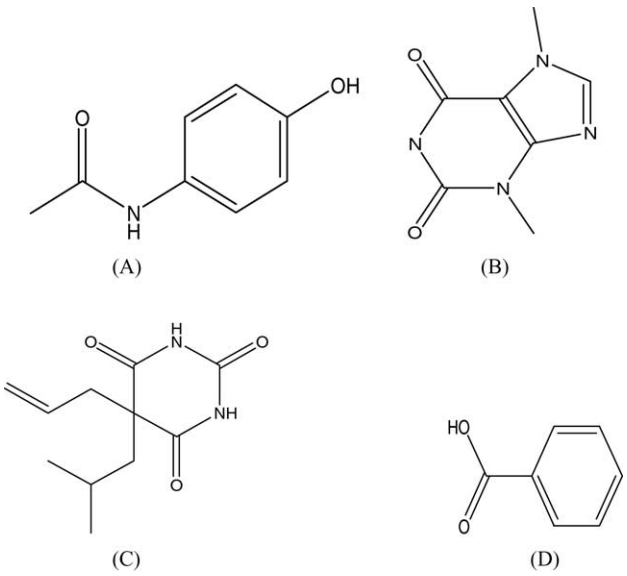

Fig. 1. Chemical structures of (A) acetaminophen, (B) caffeine, (C) butalbital, (D) benzoic acid.

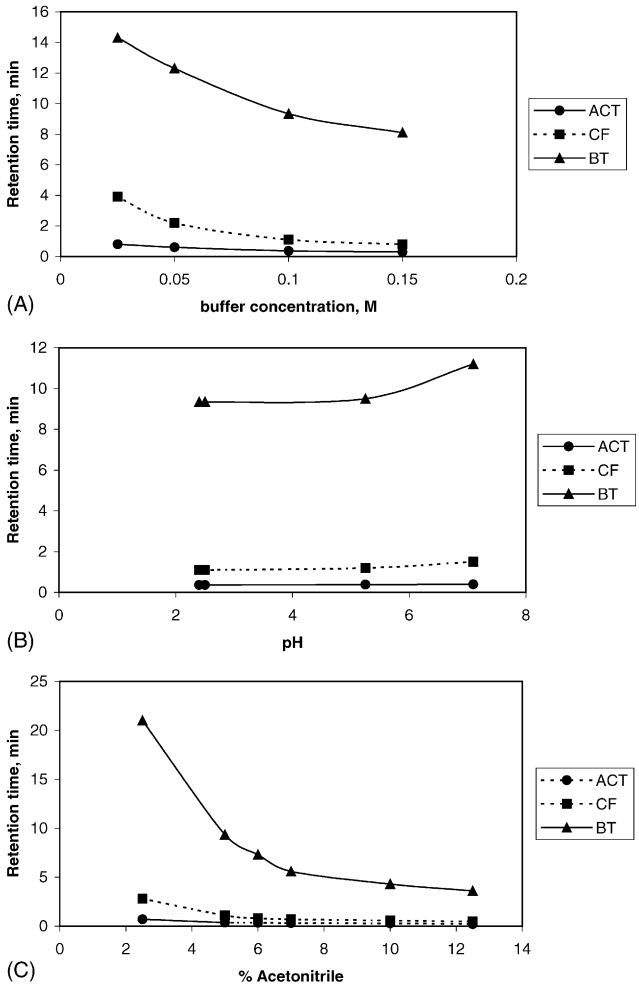

Fig. 2. Effect of (A) buffer concentration, (B) pH and (C) % ACN on the retention time of acetaminophen (ACT), caffeine (CF) and butalbital (BT).

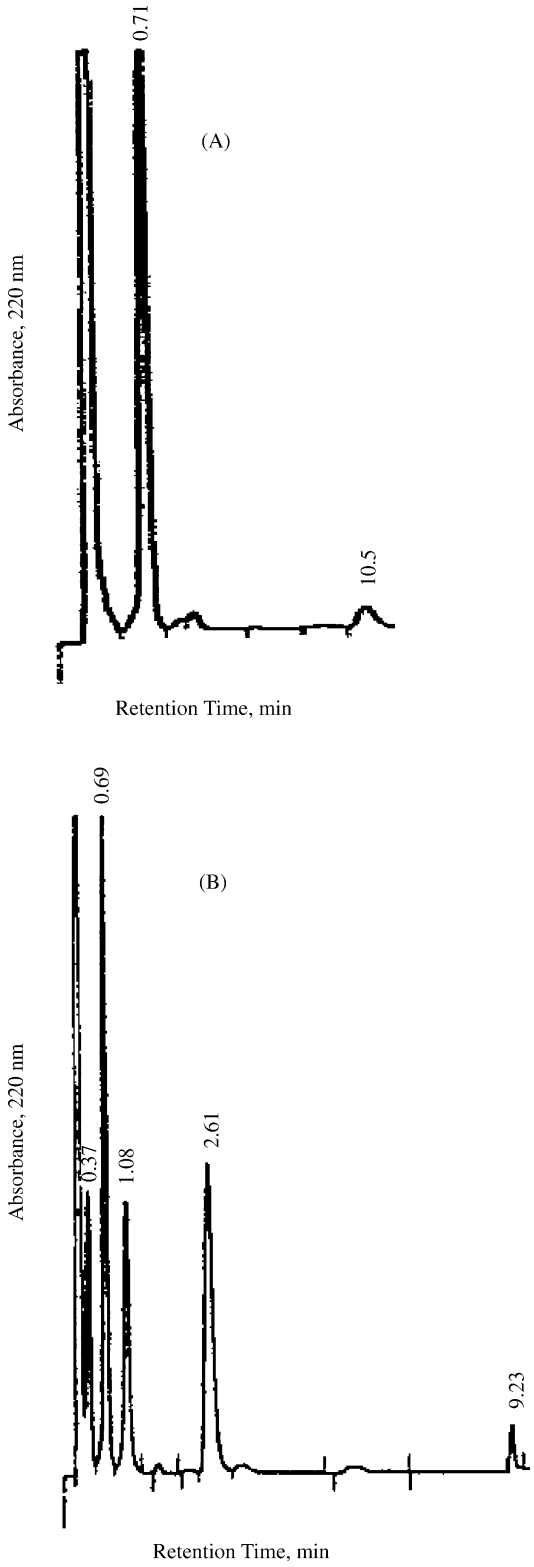

Fig. 3. Typical chromatograms of (A) blank serum and (B) spiked serum containing 12.5 μg/ml each of acetaminophen (0.37 min), caffeine (2.61 min), butalbital (9.23 min) and benzoic acid (1.08 min). HPLC conditions: monolithic column; mobile phase was 5:95 acetonitrile–0.1 M potassium phosphate monobasic buffer, pH 2.41; flow rate of 9 ml/min; detection at 220 nm; injection volume of 20 μl; column at ambient temperature (23 °C).

peaks at lower than pH 2.75 values which consequently led to a separation failure (Fig. 2B). The retention times for all the analytes were longer as the percentage of acetonitrile in the mobile phase was decreased. The result was more significant in the case of the lipophilic molecule butalbital (Fig. 2C).

The best separation of acetaminophen, caffeine and butalbital on the monolithic column was achieved using a mobile phase of 5:95 (v/v) acetonitrile 0.1 M potassium phosphate buffer pH 2.41 with retention times of 0.37 min for acetaminophen, 2.61 min for caffeine and 9.23 min for butalbital. The internal standard (benzoic acid) was well resolved with a retention time of 1.08 min. Lisinopril, promethazine, pravastatin and simvastatin elute at 0.8, 2.55, 0.66 and 1.7 min, respectively, and do not interfere with retention times of acetaminophen–caffeine–butalbital. Diazepam and lorazepam elute at 0.6 and 1.7 min, respectively, and thus interfere with the retention of the drug mixture. No interferences were observed in drug-free human serum samples. The flow rate of 9 ml/min did not yield a higher back pressure than 2600 psi and allowed a fast separation (<10 min). The detec-

Table 1
Intra- and interday accuracy and precision assay for acetaminophen (ACT)–caffeine (CF)–butalbital (BT) in serum

	Concentration added (μg/ml)	Concentration found (μg/ml)	% Error	%RSD
Intraday				
ACT	3.75	3.2 ± 0.2	−14.6	6.3
	10	9.2 ± 0.5	−8	5.4
	15	13.7 ± 1.2	−8.6	8.8
	20	17.8 ± 2.2	−11	12.4
	75	87 ± 19	16	21.5
CF	3.75	3.7 ± 0.3	−1.3	8
	10	9.7 ± 1.3	−3	13.4
	15	14.5 ± 1.8	−3	12.4
	20	19.5 ± 1.4	−2.5	7
	75	87.9 ± 17.8	17.2	20.2
BT	3.75	3.7 ± 0.3	−1.3	8.1
	10	9.8 ± 1.2	−2	12.2
	15	14.8 ± 1	−1.3	6.7
	20	19.7 ± 1.3	−1.5	6.6
	75	86.63 ± 16.98	15.5	19.6
Interday				
ACT	3.75	3.4 ± 0.1	−9.3	2.9
	10	9.2 ± 0.3	−8	3.2
	15	13.9 ± 0.9	−7.3	6.4
	20	17.8 ± 2.0	−11	11.2
	75	86.85 ± 18.41	15.8	21.2
CF	3.75	3.6 ± 0.4	−4	11
	10	9.6 ± 1.2	−4	12.5
	15	14.1 ± 1.5	−6	10.6
	20	19 ± 1	−5	6.3
	75	88.13 ± 17.98	17.5	20.4
BT	3.75	3.7 ± 0.3	−2.6	8.2
	10	9.6 ± 1.2	−4	12.5
	15	14.6 ± 1.0	−2.6	6.8
	20	19.7 ± 1.4	−1.5	7.1
	75	86.77 ± 17.09	15.7	19.7

Table 2
Recovery results for acetaminophen (ACT)–caffeine (CF)–butalbital (BT)–benzoic acid (BZ) in serum

	Concentration added (μg/ml)	Concentration found (μg/ml)	% Error	%R.S.D.
ACT	3.75	4.2 ± 0.2	10.6	4.78
	20	22.3 ± 1.1	11.45	4.9
	60	66.9 ± 2.6	11.5	3.88
CF	3.75	3.9 ± 0.3	4	6.4
	20	20.9 ± 1.4	4.5	6.7
	60	62.8 ± 2.2	4.6	3.5
BT	3.75	3.78 ± 0.19	0.8	5
	20	20.2 ± 1.4	1	6.9
	60	60.6 ± 2.4	1	3.96
BZ	3.75	4.5 ± 0.3	20	9.1

tor was adjusted at 220 nm to obtain an optimized separation in terms of the lipophilicity and sensitivity of the analytes. Phosphoric acid was added to lower the mobile phase pH. Fig. 3A and B shows chromatograms of a blank serum sample and a spiked serum sample, respectively.

Calibration plots for the analytes in serum were prepared by diluting stock solutions with filtered serum and phosphoric acid to yield concentrations of 1.25–100 μg/ml (1.25, 2.5, 7.5, 12.5, 17.5, 25, 30, 45, 65, 75, 85 and 100 μg/ml). Calibration standards at each concentration were extracted and analyzed in duplicate. Calibration curves were constructed using ratios of the observed analyte peak height to internal standard versus 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. The calibration curves showed good linearity in the range of 1.25–100 μg/ml for each analyte. The correlation coefficient (r), slope and intercept of calibration curves were 0.9996, 3.47 and 0.04 for acetaminophen, 0.998, 12 and −0.16 for caffeine and 0.995, 2.14, 0.25 for butalbital, respectively, as determined by least square analysis.

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 −14.6 to −1.3%) and precise (ranged from 2.9 to 13.4%). The acceptance criteria %RSD's of <15% and an accuracy between 85 and 115% were met in all cases.

The absolute recoveries of acetaminophen, caffeine, butalbital and benzoic acid from serum were assessed at three concentrations (3.75, 20 and 60 μg/ml). 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 compound, at each concentration, was computed using the following equation: absolute recovery = (mean peak height in extract)/(mean peak height in eluent) × 100. The results were satisfactory. The mean absolute recoveries were 89.7 ± 3.6% for acetaminophen, 95.5 ± 4.5% for caffeine, 99 ± 5.2% for butalbital and 83.4 ± 3.9% for benzoic acid (internal standard). A summary of the recovery results is given in Table 2. The LODs were 1.25 μg/ml (s/n > 3) and the LOQs were 3.75 μg/ml (s/n > 10) for each drug, respectively.

4. Conclusions

A simple method using a monolithic column was developed and validated for the determination of acetaminophen–caffeine–butalbital in human serum. The method combines a solid phase extraction procedure with a fast and sensitive isocratic reversed phase HPLC analysis with UV detection at 220 nm.

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