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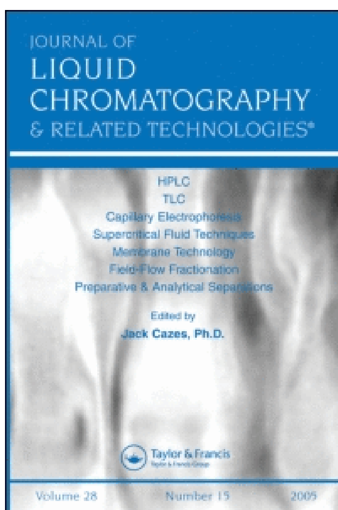

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HPLC DETERMINATION OF ANALGESICS IN HUMAN PLASMA AND SERUM BY DIRECT INJECTION ON 80 ANGSTROM PORE METHYL BONDED PHASE SILICA COLUMNS

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ABSTRACT

The feasibility of using 5 µm particle, 80 Angstrom pore sized methyl bonded phase silica columns for direct plasma and serum injection was investigated. The approach was applied to the simultaneous determination of acetaminophen and salicylic acid in human plasma. The separation was achieved using a 15 cm analytical column coupled with a 1 cm guard column of the same material and a mobile phase of 1:100 isopropanol:0.30% triethylamine in 0.05 M potassium dihydrogen phosphate (pH 6.1) with UV detection at 280 nm. The narrow pore packing gave good separation between the analytes and endogenous components. The very low organic content of the mobile phase did not induce protein precipitation. Analytical column lifetimes of approximately 140 plasma injections were obtained without loss of chromatographic resolution or excessive pressure buildup. Acetaminophen response was equivalent in plasma and serum and good correlation was shown between the direct inject HPLC approach and an enzymatic colorimetric method for acetaminophen determinations in human serum.

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INTRODUCTION

Drug and metabolite determination by direct injection of plasma or serum on an HPLC column has been extensively investigated as a means to eliminate tedious sample preparation thereby increasing sample throughput and potentially decreasing cost per assay. Shihabi (1) has recently reviewed the different approaches for direct plasma/serum injection which include micellar chromatography, internal surface reversed phase chromatography, column switching techniques and silica columns with aqueous mobile phases. In all of these approaches, sample contact with high (> 20%) concentrations of organic modifiers and/or pH extremes is avoided to eliminate protein precipitation which would rapidly plug the column. Shihabi and Dyer (2) analyzed carbamazepine in human serum by direct injection on a 300 Angstrom pore methyl bonded phase. The relatively low retention of this packing allowed the organic content of the mobile phase to be kept sufficiently low while still giving reasonably short run times. The large pore size was claimed to eliminate blocking of the pores by sample proteins.

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In the present study, an 80 Angstrom pore size, 5 µm methyl bonded phase packing was investigated for direct injection of plasma and serum. The greater surface area relative to large pore packings traditionally used for protein separations gives greater retention and higher efficiency for separations of low molecular weight species. Two model drugs: one acidic (salicylic acid, pKa = 3.0) and one basic (acetaminophen, pKa = 9.5) were chosen to demonstrate the application. Within-day and between-day assay accuracy, precision and ruggedness were validated for both analytes. In addition, good correlation between

direct injection HPLC and a commercial enzyme based colorimetric method for acetaminophen in serum was demonstrated.

EXPERIMENTAL

The chromatographic instrumentation consisted of an ACS model 351 Solvent Delivery System (Peris Industries, State College, PA), a Varian model 9090 autosampler (Varian Instrument Group, Walnut Creek, CA) equipped with a Valco injector (Valco Instruments, Houston, TX) fitted with a 10 µL injection loop, and a Shimadzu model SPD-6A variable wavelength detector (Shimadzu Instruments, Kyoto, Japan). For some experiments a Kratos model 757 variable wavelength detector (Thomson Instruments, Newark, DE) was used. A Microvax computer (Digital Equipment Corporation, Maynard, MA) with VG Multichrom software (VG Laboratory Systems, Manchester, United Kingdom) was used for data aquisition and analysis. The analytical column was a Regis 15 cm x 4.6 mm i.d. cartridge column packed with 5 µm methyl bonded phase silica, 80 Angstrom pore size (Regis Chemical Comapany, Morton Grove, IL). A 1 cm x 4.6 mm cartridge guard column packed with the same material was used with the Regis cartridge columns. A 15 cm Synchropack 300 Angstrom pore 5 µm particle methyl bonded phase column (Alltech Associates, Deerfield Park, IL) was used for some experiments. Detection was at 280 nm for all data reported. All separations were performed at ambient temperature (23-25 degrees Celsius).

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Potassium dihydrogen phosphate (Fisher), phosphoric acid and triethylamine (TEA) (both J.T. Baker) used for buffer preparation were all reagent grade. HPLC grade isopropanol (IPA), tetrahydrofuran (THF)

and acetonitrile (all J.T. Baker) were used in mobile phase preparation. In-house distilled water was purified using a Barnsted Nano-pure II (Sybron/Barnstead, Boston, MA) deionization system with an activated carbon organic removal cartridge. Acetaminophen (4-acetamidophenol) and salicylic acid (both Aldrich, reagent grade) were used to prepare standards. Acetylsalicylic acid was Aldrich, Gold Label. Neostigmine bromide (Reagent Grade, Aldrich) was used as received. Stock and spiking solutions of each drug were prepared in HPLC grade methanol (J.T. Baker). Blank serum and heparinized plasma were harvested from blood drawn from apparently healthy human volunteers. Quality Control (QC) samples were prepared at two levels for each analyte by spiking the appropriate level of drug in blank matrix. The QC pools were divided into separate aliquots and stored at -20 degrees C for use on subsequent days. Serum samples from subjects who had injested acetaminophen were obtained from York Hospital, York, PA and were stored at -70 degrees C until analyzed. All plasma samples, including matrix calibration standards and blanks, were filtered through 0.2 μm Nylon 66 filters (Micro Separations, Westborough, MA) prior to HPLC injection to remove particulate matter. For cross-validation of acetaminophen in serum, a commercial acetaminophen reagent kit (Diagnostic Chemicals Limited, Chralottetown, Prince Edward Island, Canada) was used with a COBAS/FARA centrifugal analyzer (Roche Diagnostic Systems). The commercial kit used aryl acylamide amidohydralase to convert acetaminophen to p-aminophenol. This then reacted with o-cresol (colorimetric reagent) in the presence of copper sulfate and ammonia to form a yellow indophenol derivative.

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RESULTS AND DISCUSSION

Preliminary experiments were performed using 0.05 M potassium dihydrogen phosphate buffer at pH 6.0 or 6.1 with small amounts of organic modifier as mobile phase. TEA was added as an ion pair reagent to increase the retention time of salicylic acid. Isopropanol as the organic modifier gave better separation of the analytes from each other and from endogenous interferences than did acetonitrile or THF. The TEA level was found to be the most critical mobile phase component in achieving the separation. The best separation was obtained with 1:100 isopropanol: 0.30% v/v TEA in 0.05 M potassium dihydrogen phosphate buffer at pH 6.1. This mobile phase was used for all vaidation work. While the two analytes could be resolved in analytical standards using 0.20% TEA, retention of salicylic acid decreased in plasma spikes. Apparently endogenous components interfered with the ion pairing if the TEA level was too low. Detection at 280 nm was found to give the best signal for both analytes consistent with minimal background interference. A flow rate of 1.0 mL/minute was used for all experiments. Examples of spiked and blank plasma chromatograms are shown in Figure One.

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Before validation, attempts were made to assay both acetylsalicylic acid (ASA) and its active metabolite, salicylic acid, since this would be a more realistic clinical application. ASA was well resolved for the other two analytes and eluted with a k′ of approximately 3. However, degradation of ASA to SA in spiked plasma occured rapidly due to to endogenous esterase enzymes. Such degradation has been documented to occur extensively, even in the refrigerated or frozen state (3).

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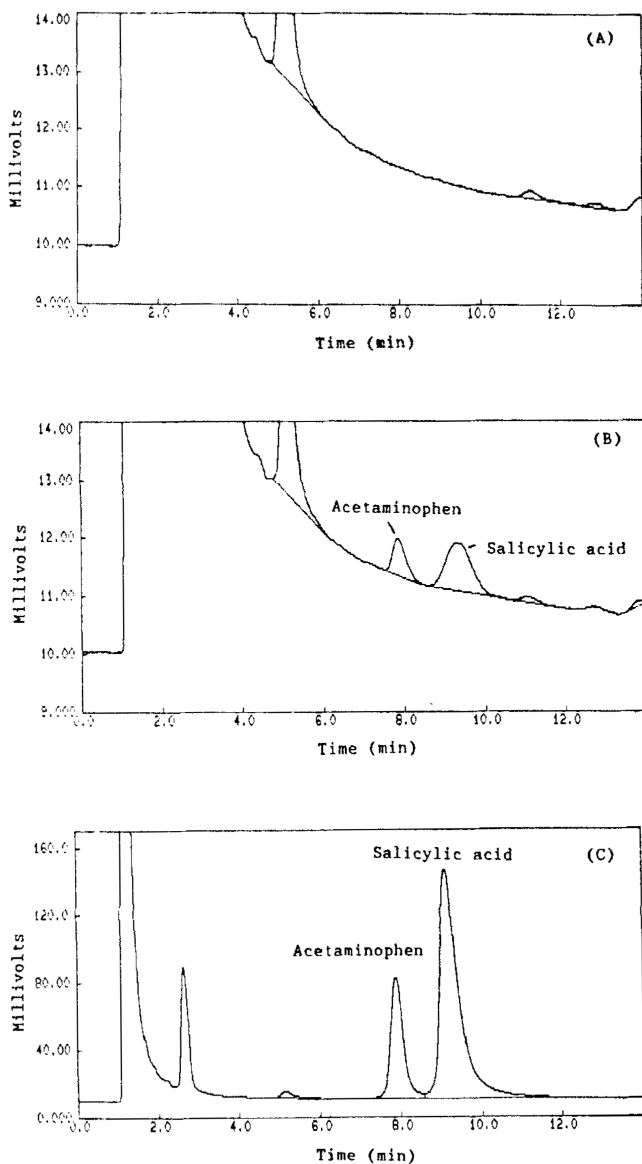

Figure One: Chromatograms of spiked and blank human plasma on 80 Angstrom pore, 5 µm methyl bonded phase silica. See text for conditions. A) blank plasma, B) plasma spiked with 5 and 10 µg/mL of acetaminophen and salicylic acid, respectively, C) plasma spiked with 500 and 1000 µg/mL acetaminophen and salicylic acid, respectively.

Attempts to eliminate this degradation using the esterase inhibitor neostigmine bromide, as reported by Sorensen (4), proved inadequate and quantitation of ASA by direct injection was deemed impractical. In standard solvent extract HPLC methods, the fresh plasma can be subjected to solvent extraction promptly after sample collection to separate esterase enzymes from the analyte. Degradation of drugs by endogenous matrix components is a potential problem common to all direct injection methods.

Validation

Table I shows the spiked plasma standard calibration curve data based on duplicate injections at each spiking level on three separate days. All results reported are based on peak heights which were found to give better precision than peak areas. For acetaminophen, calibration was based on the mean relative weight response factor (peak height divided by spiking concentration in µg/mL) calculated across the entire calibration range. The salicylic acid standards gave the best fit with a power regression of the form $y = m x$. Good precision and agreement with theory was seen for both analytes across their respective calibration ranges. Results for the QC assays are shown in Table II. Excellent precision and accuracy was demonstrated for both analytes on all three validation days. Comparison of spiked matrix standards with analytical standards prepared in mobile phase buffer showed mean absolute recoveries from plasma of 90 and 95% for acetaminophen and salicylic acid, respectively.

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TABLE I

Spiked Plasma Calibration Standard Results

Analyte	Conc. Spiked (μg/mL)	Conc. Found (μg/mL)	% RSD	% Relative Recovery
Acetaminophen	5.00	4.82	6.3	96
	10.0	9.50	2.6	95
	20.0	19.9	1.6	100
	50.0	50.8	0.9	102
	100	103	3.5	103
	200	204	3.2	102
	500	508	2.8	102
Salicylic Acid	10.0	10.2	3.3	102
	20.0	19.6	1.8	98
	40.0	39.5	4.3	99
	100	99.8	1.6	100
	200	200	2.4	100
	400	409	2.1	102
	1000	989	4.4	99

Pooled results for duplicate injections at each level per run over three separate days.

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TABLE II

Plasma Quality Control Pooled Precision and Accuracy

	Assay (µg/mL)			Pooled Estimate of Precision and Recovery
	Day 1	Day 2	Day 3	
Acetaminophen Low Control (Theory = 20.0 µg/mL)	19.4	18.6	18.5	
	19.3	18.9	18.4	
Mean	19.4	18.8	18.5	18.9
% RSD	0.4	1.1	0.5	2.2
% Recovery	97	94	92	94
Acetaminophen High Control (Theory = 100 µg/mL)	98.1	94.8	98.9	
	98.1	97.1	98.5	
Mean	98.1	95.9	98.7	97.6
% RSD	0.0	1.7	0.3	1.5
% Recovery	98	96	99	98
Salicylic Acid Low Control (Theory = 40.0 µg/mL)	39.8	37.5	38.5	
	39.9	38.5	38.3	
Mean	39.8	38.0	38.4	38.8
% RSD	0.2	1.9	0.4	2.4
% Recovery	100	95	96	97
Salicylic Acid High Control (Theory = 200 µg/mL)	204	196	197	
	204	202	197	
Mean	204	199	197	200
% RSD	0.0	2.0	0.3	1.9
% Recovery	102	100	99	100

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Column Maintenance

System backpressure under the chromatographic conditions given above was typically 1200-1300 psi. Slight increases on the order of 100-200 psi would usually occur during the course of a run. After each run of plasma samples, the columns were flushed with approximately 20 mL of water followed by a similar volume of 20% acetonitrile in water. The columns were then rinsed with water on the following morning and re-equilibrated with fresh mobile phase. The second aqueous rinse was needed to avoid precipitation of buffer salts which would occur on switching directly from 20% acetonitrile to mobile phase. Following this treatment, backpressure returned to near its original value. During initial method development work, a column set was flushed with neat acetonitrile after the first water rinse. The backpressure rose rapidly to greater than 3000 psi and the columns were irreversibly damaged. Presumably this was due to adsorbed proteins which were denatured by the acetonitrile. This did not occur when high acetonitrile concentrations were avoided using the stepwise cleanup described above, which can easily be automated on a gradient system. In this study, the guard column was changed after 70 plasma injections. Loss of chromatographic resolution and irreversible increase in backpressure were observed after an analytical column was used with two guard columns (approximately 140 plasma injections total). A new column set was then installed. The use of longer guard columns may prolong the useful life of the analytical column. Even with the relatively limited column lifetime observed, the cost of all materials on a per assay basis was approximately $3.00 which is reasonable considering the

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elimination of labor intensive sample preparation with direct injection techniques.

Retention Comparison to Wide Pore Packing

To assess the effect of packing pore size for this application, the separation was performed on a 300 Angstrom pore Synchropack methyl bonded phase column of the type used by Shihabi and Dyer for carbamazepine serum assay (2). As expected, retention of acetaminophen and salicylic acid was much less on the wider pore packing. The analytes coeluted with a k′ of about 0.9 when chromatographed using the 1:100 IPA:potassium dihydrogen phosphate/TEA buffer mobile phase. Both compounds eluted in less than four minutes at 1.0 mL/minute using 0.05 M potassium dihydrogen phosphate (pH 6.1) either with or without TEA. Increasing the buffer strength to 0.1 M did not increase retention and in all cases significant endogenous interferences were observed in plasma due to the close proximity of the analytes to the solvent front. It therefore appears that the smaller pore, higher surface area methyl bonded phase may be more applicable to polar small drug molecules than the less retentive wide pore C1 packings.

Cross-Validation

The direct injection HPLC approach for acetaminophen was cross-validated against a commercial enzyme colorimetric procedure using a COBAS centrifugal analyzer. QC samples were prepared using pooled serum to verify the eqivalence between plasma and serum samples using the HPLC assay. Excellent precision and accuracy were

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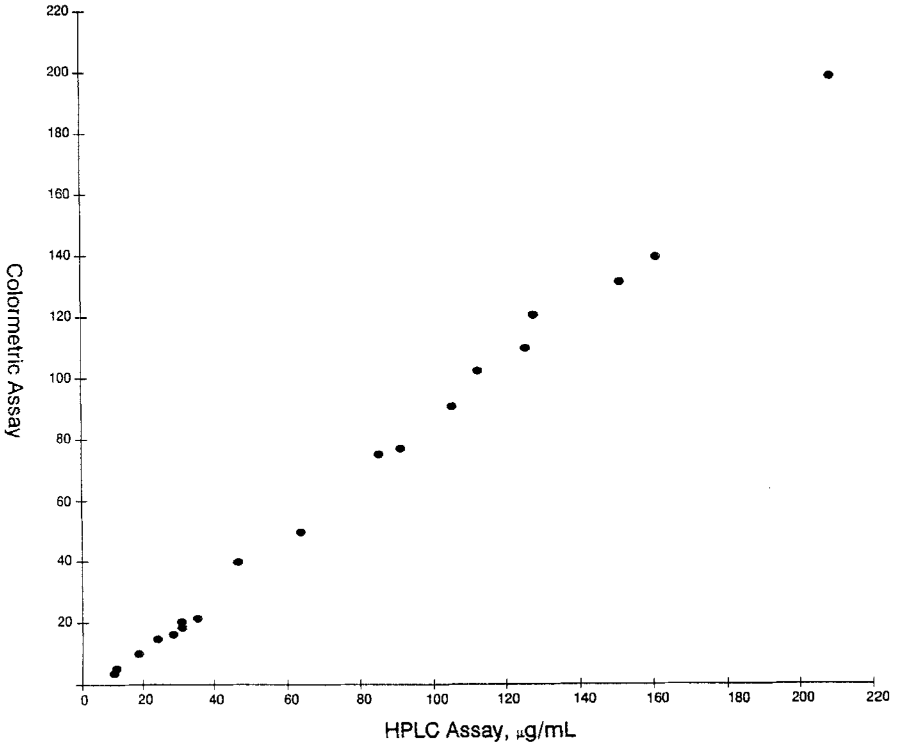

Figure Two: Comparison of serum assays from acetaminophen dosed subjects by direct injection HPLC (x-axis) and enzyme colorimetric procedure (y-axis). See text for experimental conditions.

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demonstrated. Blank serum from three normal subjects showed no endogenous interferences. Twenty one serum samples from subjects who had injested acetaminophen were analyzed by both HPLC and colorimetry and the colaborative data are shown in Figure Two. Linear regression analysis of the data gave a fit of $y = 0.9562\ x - 6.186$ with a correlation coefficient of 0.997. While excellent correlation was demonstrated across the concentration range, a slight bias was evident

between the two methods with HPLC values being somewhat higher for a given sample than those obtained with the colorimetric assay. Since endogenous chromatographic interferences were not observed, there is no apparent explanation for this difference. In any case, a bias of this magnitude would not be clinically significant.

CONCLUSIONS

The applicability of 80 Angstrom pore methyl bonded phase silica columns for the direct determination of drugs in plasma and serum has been demonstrated. While column life under these conditions was not as great as that reported for some direct-injection HPLC approaches, the per assay cost and time interval between guard column changes (maximum run length) were reasonable. The high retention for polar small molecules observed with the narrow pore material can be an advantage when assaying polar drugs or metabolites.

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