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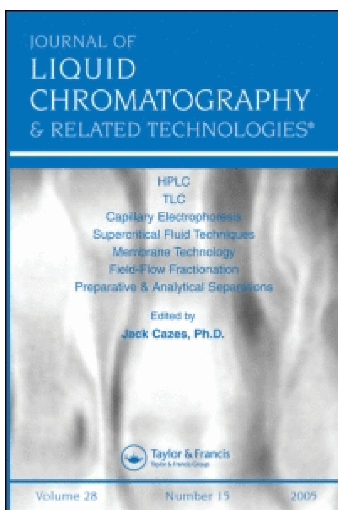

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Direct Injection Analysis of Diuretic and Anti-Inflammatory Drugs on a Shielded Hydrophobic Phase Column

C. T. Santasania[a]
[a] Supelco, Inc. Division of Rohm and Haas Supelco Park Bellefonte, Pennsylvania

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DIRECT INJECTION ANALYSIS OF DIURETIC AND ANTI-INFLAMMATORY DRUGS ON A SHIELDED HYDROPHOBIC PHASE COLUMN

C. T. SANTASANIA
Supelco, Inc.
Division of Rohm and Haas
Supelco Park
Bellefonte, Pennsylvania 16823

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ABSTRACT

A shielded hydrophobic phase (SHP) column was used for the direct injection analysis of drugs from serum. Gradient elution gave the best separation; however, an isocratic separation is possible. The mobile phase pH was found to play a major role in the separation; ionic strength had a lesser influence. Quantitation of the drugs of interest was possible over several orders of magnitude. Inter- and intra-day recoveries showed values near 100%. A drug-containing serum sample was analyzed, using the described method, and the results were compared to a related liquid chromatographic method.

INTRODUCTION

Determination of drugs in biological fluids has posed numerous analysis problems for analysts using liquid chromatography. Physiological fluids such as serum or urine contain a large number of proteinaceous and other compounds that may interfere with analyte determination by direct injection. In addition, direct injection of complex

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biological samples often leads to precipitation and adsorption of matrix components onto the column. This can result in increased column backpressure, chromatographic band broadening, and reduced overall column lifetime.

To alleviate the problems associated with direct injection techniques, several alternatives have been suggested. These include direct injection of only small volumes (<3μl) of biological fluids directly onto the column, followed by washing (1); column switching techniques (2,3,4) and, most recently, the use of micellar mobile phases (5,6,7) to solubilize proteins. These methods, their advantages, and their disadvantages have been described and reviewed extensively (8).

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In contrast to the techniques mentioned above, a number of investigators have been pursuing the development of unique chromatographic stationary phases for the direct injection of untreated biological samples. Yoshida et al. (9) prepared a protein-coated C_{18} column which retained the characteristics of the C_{18} column for small hydrophobic molecules, but allowed for the exclusion of plasma proteins. Pinkerton et al. (10) described the preparation of an internal surface reversed-phase (ISRP) material consisting of a hydrophobic oligopeptide bonded to 80Å pore silica gel. An enzymatic cleavage with a proteinase produced a packing material with hydrophobic ligands on the external surface and internal surfaces with the uncleaved hydrophobic oligopeptide. The liability of the ISRP material lies in the narrow pH range (11) that must be used, since protein

exclusion is based on a pH dependent electrostatic repulsion mechanism.

Gisch et al. (12) prepared a shielded hydrophobic phase on 100Å silica. This material consists of enclaved hydrophobic phenyl groups in a polymeric hydrophilic network of polyethylene oxide. The hydrophilic network of the bonded phase forms a water solvated interface that allows for the passage of small analytes, which are retained by reversed-phase interactions. Larger water solvated proteins are prevented from interacting with the hydrophobic regions by shielding provided by the hydrophilic groups. Further details of the surface structure of this phase have been described (13). This unique dual phase allows for the direct injection of physiological fluids, such as serum. The chromatographic result is the elution of the protein matrix unretained at or near the column void volume, with retention of the smaller analytes of interest.

In this study, we applied the concept of the shielded hydrophobic phase to the determination of a number of diuretic, anti-inflammatory, and anti-hypertensive drugs directly from serum.

Hydrochlorothiazide, furosemide, oxyphenbutazone, and phenylbutazone are commonly used in both human (14-26) and veterinary medicine (27-33). These drugs are also used extensively in the horse racing industry (27,28,30,32,33). Hydrochlorothiazide is a diuretic and anti-hypertensive. It is usually administered along with other, complimentary,

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anti-hypertension agents. As with all diuretics, it increases excretion of both sodium and chloride (14).

Phenylbutazone is a non-steroidal, anti-inflammatory drug. Its primary metabolite, oxyphenbutazone, exhibits anti-pyretic and analgesic properties. It is believed that phenylbutazone inhibits certain factors associated with the inflammatory process. These processes include prostaglandin synthesis, red blood cell migration, and release and activity of lysosomal enzymes. It is prescribed for gout, rheumatoid arthritis, and degenerative joint disease of the hips and knees (14).

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Furosemide, a diuretic, is an anthranilic acid derivative. It is indicated for the treatment of edema caused by congestive heart failure, cirrhosis of the liver, and renal disease. It is also an anti-hypertensive compound (14).

Monitoring of these compounds is necessary by clinical labs because of the widespread use of these drugs as anti-hypertension agents. Veterinary and regulatory labs need to monitor these drugs for both therapeutic and forensic reasons.

A variety of liquid chromatographic methods have been developed (15-26,28-33) to quantify these drugs from serum or plasma. Unfortunately, these methods involve extensive sample clean-up to remove proteins before high performance liquid chromatography (HPLC) analysis. Pinkerton (34) reported on a direct injection technique for some of these compounds. This method, however, does not allow for the

determination of hydrochlorothiazide. In addition, pH limitations of the column restrict the usefulness of the method.

The work described here uses a direct injection technique, without any prior sample clean-up. In addition to developing a separation on the shielded hydrophobic phase column, factors such as pH and ionic strength of the mobile phase were investigated to determine their effect on the separation. Linearity data as well as sample recovery are reported. Finally, a serum sample from an infused horse was analyzed and quantitated for phenylbutazone.

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EXPERIMENTAL

Chromatographic System

The liquid chromatograph consisted of a Waters (Milford, MA, USA) 501 HPLC pump and a Waters 590 HPLC pump plumbed for producing a high pressure gradient. A Waters Wisp 712 Automatic Sample Injection System was used for sample introduction. Detection was accomplished with a Kratos (Applied Biosystems, Ramsey, NJ, USA) Spectroflow 757 Absorbance Detector. A Waters 720 System Controller was used to control the pumps and autosampler. All data was recorded on a Waters 745B Data Module run at a chart speed of 1.0cm/min.

Columns

A Hisep Shielded Hydrophobic Phase column (Supelco, Inc., Bellefonte, PA, USA) (5μ packing, 15cm x 4.6mm ID) was

used throughout this study. Two additional Hisep columns from different bonding and silica lots were used for the batch-to-batch reproducibility study. An in-line 0.5μ frit filter (Supelco) was used to protect the analytical column.

Mobile Phases, Reagents and Drugs

Acetonitrile, methanol, and ammonium acetate were obtained as HPLC grade from J.T. Baker (Phillipsburg, NJ, USA). Reagent grade ammonium hydroxide and acetic acid were also from J.T. Baker. Purified water (18 megaohm) was obtained from a Milli-Q System (Millipore Corp., Bedford, MA, USA) and was used in the preparation of all samples and mobile phases. Buffers were filtered through 0.45μ Magna Nylon 66 membrane filters (Supelco) and all mobile phases were degassed under vacuum in an ultrasonic bath before use.

The A (weak) component of a gradient mobile phase, 180mM ammonium acetate, was prepared in water. The pH of this solution was adjusted with 180mM acetic acid (aq) to a pH of 5.4. All pH measurements were made using a Fisher Accument pH meter (Fisher Scientific, Pittsburgh, PA, USA) equipped with a standard pH electrode. Acetonitrile was the B (strong) mobile phase component. Gradient conditions listed in Table 1 were used for all experiments, unless stated otherwise.

For the pH study, mobile phase A was prepared by diluting concentrated ammonium hydroxide and acetic acid to 180mM with water. The 180mM acetic acid was titrated with the 180mM ammonium hydroxide to give the desired pH. A pH range of 4 to 7 was used in this study.

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TABLE 1

Gradient Used for Separation of Diuretics and Anti-Inflammatory Drugs

Time	Flow Rate	%A	%B
0	2.0	80	20
5	2.0	70	30
10	2.0	70	30
12	2.0	80	20

Linear gradient between 0 and 5 min., hold for 5 min. and return to initial conditions over 2 min.

Mobile Phase: A= 180mM ammonium acetate (aq) pH 5.4 with 180mM acetic acid
B= acetonitrile

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In the ionic strength study, mobile phase A was prepared by diluting a 500mM stock solution of ammonium acetate to 250, 180, 125 and 62.5mM with water. At each ionic strength, the pH was adjusted to 5.4 with acetic acid of the same ionic strength as the mobile phase (e.g., 125mM ammonium acetate was adjusted to pH=5.4 with 125mM acetic acid). In both the pH and ionic strength experiments, the gradient listed in Table 1 was used.

Furosemide and hydrochlorothiazide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Phenylbutazone, USP, and oxyphenbutazone, USP were obtained from the United States Pharmacopeial Convention, Inc. (Rockville, MD, USA).

Triple filtered fetal bovine serum was obtained from Hyclone Labs (Logan, UT, USA).

Control horse serum and horse serum taken 4 hours after administration of a 2g infusion of phenylbutazone were generously donated by R. McKenzie of the Michigan Department of Agriculture, East Lansing, MI, USA.

Stock solutions of each drug standard were prepared in methanol at 1mg/ml. These stock solutions were then diluted 1:1 with water. Appropriate dilutions to the desired concentrations were made with water or bovine serum. The phenylbutazone containing horse serum and control horse serum were used as received. All samples were stored refrigerated when not in use.

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METHODS

Batch-to-batch Reproducibility Study

Three columns, representing three bonding lots and two silica batches, were studied. Mixtures of the four test compounds were analyzed in triplicate on each column. The k' values were determined using uracil as a V_0 marker.

Linearity Study

The responses of furosemide and phenylbutazone were investigated, using two separate calibration ranges. The higher range, 200 to 25μg/ml, would be the range used for human therapeutic work. The lower values, between 2 and 0μg/ml, were chosen because this range would be of interest to regulatory agencies. Calibration curves were run with

the sample spiked into water and into serum. Three injections of each sample were made in all cases.

Recovery Study

A study of the amount of drug recovered from a spiked serum sample was undertaken over a three-day period. Three levels of spiking were investigated: 1μg/ml, 10μg/ml, and 100μg/ml. Furosemide and phenylbutazone were the drugs studied, and each was investigated separately. Both water and serum were spiked at each level. Each sample was injected four times, over the three-day period.

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Horse Serum Sample

A calibration curve was prepared for quantitating this sample. Standards in the range from 15 to 0μg/ml were prepared in the control horse serum. Three injections of each standard concentration and three injections of the actual sample were made.

RESULTS AND DISCUSSIONS

Chromatographic Analysis

Figure 1 shows the structures of the drugs used in this study. Previous work (35) with these four compounds indicated that isocratic elution of the compounds, individually, was possible on the Hisep column. An isocratic separation using 180mM ammonium acetate (pH=7) with 20% acetonitrile gave incomplete resolution of furosemide and oxyphenbutazone. Decreasing the pH of the mobile phase to 5.4 gave complete resolution of the four

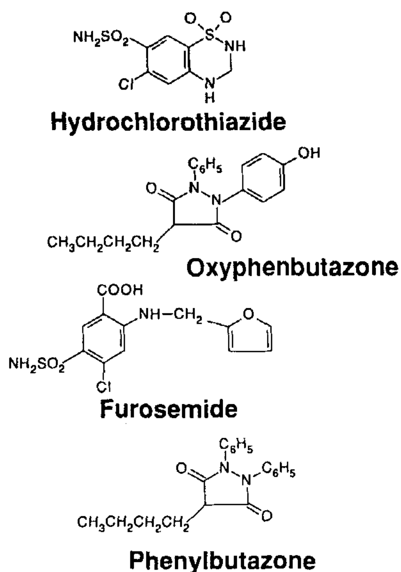

FIGURE 1. Drugs Used in This Study.

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compounds, as shown in Figure 2. The serum peaks elute unretained, followed by hydrochlorothiazide, oxyphenbutazone, furosemide, and phenylbutazone. This separation is not optimal, as indicated by the long delay time between hydrochlorothiazide and oxyphenbutazone. In addition, oxyphenbutazone, furosemide, and phenylbutazone peaks are very broad, with sufficient space between bands to indicate that a gradient separation should be possible.

Figure 3 shows the optimized gradient separation of the four compounds, using the gradient shown in Table 1. The separation is accomplished in much less time than in Figure 2, even with the five minute equilibration delay. Clearly,

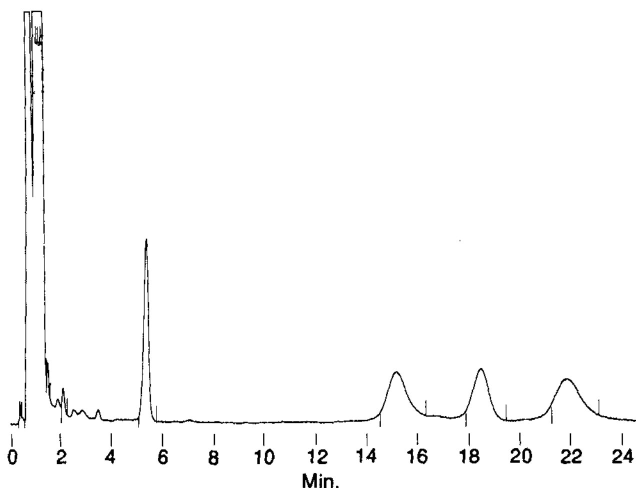

FIGURE 2. Isocratic Separation of Diuretics/Anti-Inflammatory Drugs, pH=5.4. Chromatographic Conditions: Isocratic Separation. Column: Hisep 15cm x 4.6mm ID. Mobile phase: 180mM Ammonium Acetate, pH=5.4/Acetonitrile, 80/20. Flow rate: 2.0ml/min. UV at 267nm, 0.016 AUFS. Temperature: 30°C. Injection volume: 10µl. Sample concentrations/elution order: 5µg/ml Hydrochlorothiazide, 10µg/ml Oxyphenbutazone, Furosemide, Phenylbutazone (in serum).

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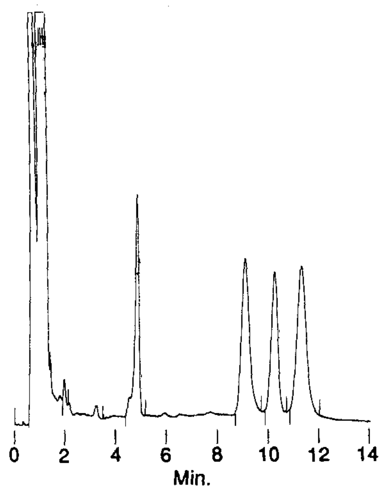

FIGURE 3. Optimized Gradient Separation of Diuretics/Anti-Inflammatory Drugs. Gradient Conditions listed in Table 1. Column: Hisep 15cm x 4.6mm ID. UV at 267nm, 0.016 AUFS. Temperature: 30°C. Injection Volume: 10µl. Sample concentrations: 5µg/ml hydrochlorothiazide, 10µg/ml oxyphenbutazone, furosemide, phenylbutazone (in serum).

TABLE 2

Batch-to-Batch Reproducibility Study of Hisep Material for Diuretics/Anti-Inflammatory Drugs Analysis

Column#	Bonding Lot	Silica Lot	k'_{Hyd}	k'_{Opb}	k'_{Fur}	k'_{Pbu}
207039AA	1232	A	3.78	7.95	9.28	10.14
207190	1202	A	3.58	7.45	8.66	9.55
207110	1165	B	3.59	8.33	9.83	10.73
Between the three columns:		X =	3.65	7.91	9.26	10.14
		SD =	0.092	0.360	0.477	0.481
		RSD =	2.52%	4.55%	5.16%	4.75%

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the gradient separation offers distinct advantages over the isocratic method in terms of both time and chromatographic efficiency.

Another useful aspect of the Hisep column is illustrated in Figure 3. After elution of the serum peaks at the void volume, any concentration of organic modifier can be used to elute the analyte peaks without concern for precipitation of the serum proteins, as has been the case in other direct injection techniques (1-4).

BATCH-TO-BATCH REPRODUCIBILITY STUDY

Possibly the most important question about any method is whether or not the column used gives reproducible results

from one production batch to the next. This was investigated in this study. The gradient conditions listed in Table 1 were used with all columns. After calculating k' values for three different batches of Hisep materials, the results were tabulated and are shown in Table 2. The maximum relative standard deviation (rsd) was about 5% for the three batches of materials studied, indicating good inter-batch reproducibility.

EFFECT OF pH AND IONIC STRENGTH ON THE SEPARATION

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The pH vs. k' data are shown in Figure 4. Constant ionic strength at 180mM was maintained throughout this part of the study. Furosemide is the compound most profoundly affected by pH. Figure 4 shows that the pH value of 5.4 is the optimum for separation of these four compounds. At pH values of 6 and above, oxyphenbutazone and furosemide will co-elute. At pH values less than 5.4, the resolution between furosemide and phenylbutazone decreases dramatically, and k' values for furosemide increase. Under these conditons, the carboxylic group of the furosemide molecule becomes protonated and enhances the molecule's hydrophobicity. Mobile phase pH has the greatest influence on the ionization of the furosemide, with the pH affecting the k' values of the oxyphenbutazone and phenylbutazone only slightly. No effect on the k' of the hydrochlorothiazide is seen over the pH range studied. Figure 4 reveals much about the importance of pH in this separation.

Figure 5 shows the effect of salt concentration on k' at a constant pH of 5.4. Selectivity in this separation was

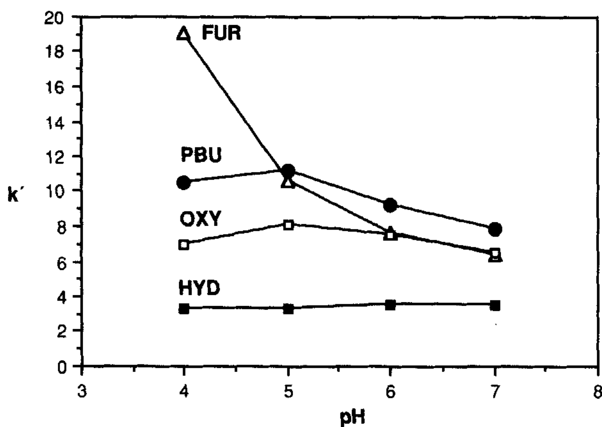

FIGURE 4. Effect of pH on k', Using Constant Ionic Strength Buffer (180mM Ammonium Acetate). Conditions listed in the text.

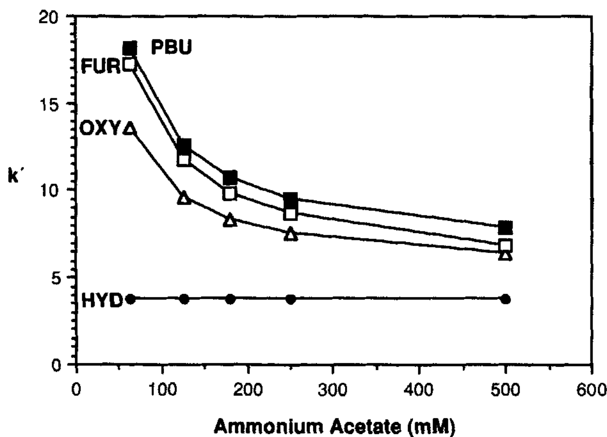

FIGURE 5. Effect of Ionic Strength on k', Using Constant pH Buffer (pH 5.4). Conditions listed in the text.

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not influenced by ionic strength. The k' values, however, decreased with increasing ionic strength. Furosemide, oxyphenbutazone, and phenylbutazone behave like other acidic compounds that have been studied using the Hisep column in our lab. The 180mM ammonium acetate mobile phase gave optimal k' values (36) between 4 and 12.

LINEARITY STUDY

The linear range of a method becomes important if reliable quantitation is desired. Tables 3 and 4 show calibration data for furosemide and phenylbutazone. Both drugs showed linearity over three orders of magnitude, with the intercept passing essentially through zero. The drugs were spiked into both water and serum because we were interested in the response of the drug in both matrices. Analysis of variance tests on the slopes indicated no differences between the line obtained with the serum-spiked sample and the water-spiked sample. Slope responses for the compounds of interest were independent of the matrix, as would be expected. Excellent values were also obtained for r^2, the amount of variation accounted for by the equation of the line.

RECOVERY STUDY

Tables 5 and 6 show the results of a recovery study for furosemide and phenylbutazone performed over a three-day period, at three different drug concentrations. Recovery

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TABLE 3

Summary of Best-Fit Lines for Furosemide as Determined by Least Squares Method

Drug Level	Matrix	Best Fit Line	(r^2)*
0.25-2μg/ml	Water	y=1.750 x + (-)0.016	0.999
0.25-2μg/ml	Serum	y=2.000 x + 0.274	0.993
25-200μg/ml	Water	y=0.061 x + 0.000	0.999
25-200μg/ml	Serum	y=0.072 x + (-)0.241	0.999

*r^2 = amount of variation accounted for by the equation.

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TABLE 4

Summary of Best-Fit Lines for Phenylbutazone as Determined by Least Squares Method

Drug Level	Matrix	Best Fit Line	(r^2)
0.25-2μg/ml	Water	y=1.960 x + 0.059	0.999
0.25-2μg/ml	Serum	y=2.220 x + 0.132	0.995
25-200μg/ml	Water	y=0.073 x + (-)0.383	0.993
25-200μg/ml	Serum	y=0.073 x + (-)0.185	0.999

TABLE 5

Recovery Study: Furosemide in Serum[1]

	1μg/ml	10μg/ml	100μg/ml
Day 1			
% Recovered	100.0	100.0	110.0
SD	0.021	0	0.054
RSD	0.58%	0%	0.37%
Day 2			
% Recovered	96.4	104.0	92.9
SD	0.061	0.047	0.198
RSD	1.85%	3.22%	1.43%
Day 3			
% Recovered	96.2	100.4	100.7
SD	0.235	0.034	0.216
RSD	7.70%	2.95%	1.75%
Mean for Three Days			
% Recovered	97.5	101.4	101.2
SD	0.105	0.027	0.156
RSD	3.37%	2.06%	1.19%

[1] n=4 analyses

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TABLE 6

Recovery Study: Phenylbutazone in Serum[1]

	1μg/ml	10μg/ml	100μg/ml
Day 1			
% Recovered	85.0	103.0	109.0
SD	0	0.025	0.151
RSD	0%	1.54%	1.56%
Day 2			
% Recovered	84.0	100	102.5
SD	0.043	0	0.081
RSD	1.58%	0%	1.02%
Day 3			
% Recovered	83.0	101.8	104.2%
SD	0	0.021	0.064
RSD	0%	3.03%	0.80%
Mean For Three Days			
% Recovered	84.0%	101.6	105.2%
SD	0.014	0.015	0.098
RSD	0.53%	1.52%	1.13%

[1]n=4 analyses

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TABLE 7

Recovery Study: Furosemide in Serum

Inter-Day (for Three Days)

Mean recovery at the three levels: 100.05%

Precision between three days: 2.20%
(average 3RSD for three days)

Intra-Day

Precision at three levels:	Day 1:	0.32%
	Day 2:	2.16%
	Day 3:	4.13%

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Recovery Study: Phenylbutazone in Serum

Inter-Day (for Three Days)

Mean recovery at the three levels: 96.95%

Precision between three days: 1.06%
(Average 3 RSD for three days)

Intra-Day

Precision at three levels:	Day 1:	1.03%
	Day 2:	0.86%
	Day 3:	1.28%

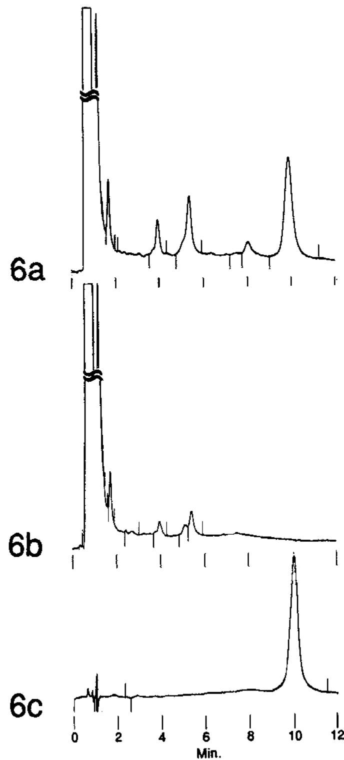

FIGURE 6. Analysis of Infused Phenylbutazone from Horse Serum. Gradient Conditions listed in Table 1. Column: Hisep 15cm x 4.6mm ID. UV at 267nm, 0.016 AUFS. Temperature: 30°C. Injection Volume: 10µl. Samples: 6a - Horse Serum taken 4 hours after a 2g infusion of phenylbutazone, 6b - control horse serum; 6c - phenylbutazone in water at 10µg/ml.

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was calculated by using the following ratio:

$$\frac{\text{peak height of serum spiked sample}}{\text{peak height of water spiked sample}} \times 100$$

Recoveries were excellent for both drugs, with relative standard deviations not greater than 3.5%. In addition,

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TABLE 8

Quantitation of Phenylbutazone in Horse Serum
Best-Fit Line and Comparison to Value Calculated by Michigan Lab.

Best-Fit Line
(this method): $y=0.429\,x + (-)0.075$ $(r^2) = 0.999$

Calculated concentration in sample: 10.45µg/ml SD=0.10

Value obtained by Michigan Lab: 9.35µg/ml

both inter- and intra-day precision studies are shown in Table 7. Recoveries of the drugs for the three-day period were between 96 and 100%, with average precision of 1-2%. Intra-day values were also very acceptable for this gradient method.

REAL SAMPLE

Figure 6a shows a chromatogram obtained after injection of serum from a horse infused with 2.0g of phenylbutazone. Figure 6b is control horse serum. No interferences from the serum are seen. Figure 6c is 10μg/ml of phenylbutazone in water, shown for retention time verification.

Quantitative analysis was performed on the horse serum sample. Table 8 shows the calibration curve used for quantitating the phenylbutazone from the horse serum. Using this curve, a value of 10.45μg/ml of the drug was determined.

A similar HPLC method, using the Hisep material, was developed by analysts at the Michigan Department of Agriculture. The horse serum sample mentioned above was quantitated using their procedure. They obtained a value of 9.35μg/ml. These numbers compare favorably, indicating the method described here is useful for real sample analysis.

The use of a shielded hydrophobic phase column described here offers certain advantages over previously described methods (1-4, 15-26, 34) for analyzing drugs in physiological fluids. In the procedure described in this paper, complex biological samples can be directly injected

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onto the column without any sample pre-treatment. Gradient separations can be used with this type of column, since all protein peaks elute at or near the void volume. Linearity and recovery using the described method are excellent. Finally, the separation can be used reliably on actual samples for drug quantitation.

Acknowledgement

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