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Direct injection HPLC method for the determination of phenylbutazone and oxyphenylbutazone in serum using a semipermeable surface column

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Abstract

A direct injection HPLC method has been developed for the determination of phenylbutazone and its active metabolite oxyphenylbutazone in serum using a semipermeable surface (SPS) column. The method is easy to perform and requires 20 μl of a filtered serum sample. The chromatographic time is less than 13 min using a mobile phase of 15:85 v/v acetonitrile-0.05M phosphate buffer pH 7.5. The method was linear in the range 0.5–20 μg ml^{-1} ($r > 0.99$, $n = 6$) with R.S.D. less than 6%. Interday and intraday variability were found to be less than 8.3%. The limit of quantitation and detection were 0.5 and 0.25 μg ml^{-1} ($s/n > 3$), respectively, for both drug and metabolite. © 1997 Elsevier Science B.V.

Keywords: High performance liquid chromatography; Phenylbutazone; Oxyphenylbutazone; Semipermeable surface column; Serum

1. Introduction

Direct injection of serum onto a conventional bonded-phase HPLC column is an attractive analytical technique that is of value for high sample throughput. Two review papers [1,2] have discussed the present status and problems of the direct injection approach. Two major problems usually associated with the technique are column plugging and deterioration of the stationary phase due to adsorption of protein or other endogenous compounds. To overcome these problems, some analysts have employed acetonitrile or methanol as a protein precipitant in a serum pretreatment step prior to centrifugation and injection of the supernatant containing the analyte onto the column. This procedure has been called 'dilute and shoot' or non-extraction method. It was used successfully in our laboratory for the assay of *p*-aminosalicylic acid at therapeutic levels in serum [3]. There is always the possibility of drug entrapment in the precipitant, but good recovery of analyte can be obtained with a gradual addition of acetonitrile or methanol while mixing the serum sample. It is also possible that endogenous

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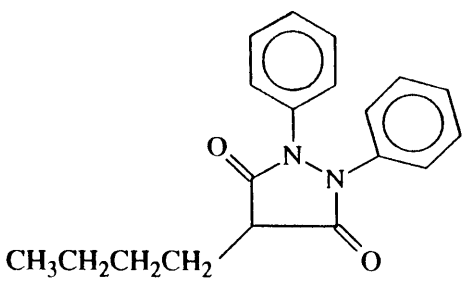
serum components which are solubilized in the organic solvent might interfere with analyte peaks, but the use of a higher efficiency HPLC column could allow for a much better separation of the peaks.

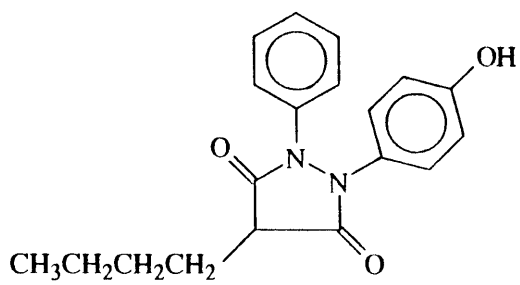
The classical methods of serum sample pretreatment such as liquid-liquid extraction or solid phase extraction are still favored by many analysts and allow excellent analyte recovery and cleaner samples, but require additional labor and time consuming steps. A number of new non-extraction techniques have been developed which were designed to incorporate the best parts of the dilute and shoot approach without protein denaturation or other manipulative steps. These major techniques are (i) direct injection using restricted access media(RAM) columns [2], (ii) column switching methods [4], and (iii) micellar chromatography [5]. The RAM approach is the simplest and uses specially designed stationary phases to restrict the interaction of serum proteins with partitioning of the analyte. Commercially available RAM columns include the Semipermeable surface (SPS), the Pinkerton or Internal surface reversed phase (ISRP) and the hydrophobic shielded phase (Hisep). The disadvantages of RAM columns are loss of column efficiency and increased back pressure over time. Column switching methods have been successfully used to overcome these problems, but need extra pumps, columns and switching valves. Micellar chromatography reduces the protein precipitation problem, but suffers from low chromatographic efficiency and sensitivity.

In this paper, a direct injection method for the determination of phenylbutazone and its active metabolite oxyphenylbutazone (Fig. 1) in serum is reported using the SPS column. Phenylbutazone is a potent nonsteroidal, anti-inflammatory drug used by veterinarians to treat livestock. Its use in humans is limited because of the side effects and the availability of newer and safer anti-inflammatory drugs. In a survey of veterinarians, it was found that about 69% use phenylbutazone to treat arthritis, mastitis, pyrexia, endotoxemia, viral respiratory disease and other inflammatory conditions in animals [6,7]. Practical uses of phenylbutazone in food producing animals have been reviewed by Kopcha and Ahl [8]. Phenylbutazone is metabolized in the liver to the pharmacologically active oxyphenylbutazone [9].

Analytical methods previously reported for phenylbutazone include HPLC [10], SFC [11], GC-MS [12], and fluorometry [13]. A number of papers have also reported that use HPLC in pharmacokinetic studies of phenylbutazone and oxyphenylbutazone in neonatal foal [14], neonatal calves [6], dogs [15], horses [10], and goats [16]. A recent literature survey indicated that there is a need for pharmacokinetic and toxicokinetic studies of phenylbutazone in neonatal animals by different routes of administration. Most of the HPLC methods used liquid-liquid extraction to isolate the analytes from serum. The direct injection method described herein requires no serum sample clean up step and should be applicable to the detection of microgram levels of phenybutazone and oxyphenylbutazone in animals. The cur-



PHENYLBUTAZONE



OXYPHENYLBUTAZONE

Fig. 1. Chemical structures.

rent maximum limit for phenylbutazone in racehorses is 2 μg ml^{-1} of plasma [10].

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Model 760 pump (Micromeritics, Norcross, GA), a Model 2250 variable wavelength UV/VIS detector (Varian, Walnut Creek, CA) and an integrator (Chromatopac Model C-R3A, Shimadzu, Kyoto, Japan). A Rheodyne Model 7125 injector (Cotati, CA) equipped with a 20-μl loop was used for injection.

2.2. Reagents and chemicals

Phenylbutazone and oxyphenylbutazone were purchased from Sigma (St. Louis, MO). Drug free serum and filters (0.2 μm, Acrodisc, polysulphone) were obtained from Instrumentation Lab (Lexington, MA) and Gelman Sciences (Ann Arbor, MI), respectively. Disposable 1-ml syringes and dibasic sodium phosphate were obtained from Becton Dickinson (Franklin Lakes, NJ) and J.T. Baker Company Inc. (Phillipsburg, NJ), respectively.

2.3. Chromatographic conditions

Separation of the drug and metabolite was achieved on the SPS column with the detector set at 265 nm and at ambient temperature (23 ± 1°C). The analytical column was protected with a commercially available guard column containing the same packing material as that of the analytical column. The mobile phase was a mixture of 15:85 v/v acetonitrile-0.05 M sodium phosphate buffer pH 7.5 and the flow rate was maintained at 1 ml min^{-1}. The semipermeable surface column (SPS-5PM-S5-100-C18, 15 cm × 4.6 mm i.d.), Pinkerton ISRP High Efficiency HPLC Column (GFF-S5-80, 15 cm × 4.6 mm i.d.) and their respective guard columns were purchased from Regis Chemical (Morton Grove, IL).

2.4. Sample preparation

A combined stock solution containing 500 μg ml^{-1} of phenylbutazone and oxyphenylbutazone was prepared in acetonitrile and stored at 4°C. Spiking solutions of 5, 10, 25, 50, 100 and 200 μg ml^{-1} were prepared by dilution of the stock with acetonitrile. Aliquots (100 μl) of the spiking solutions were added to individual tubes, dried under a nitrogen flow and the residues dissolved in 1 ml of serum to give concentrations in the 0.5–20 μg ml^{-1} range. Each tube was vortexed for 30 s and the serum filtered into a clean tube. Then, 20 μL of the filtered serum was then injected onto the HPLC column.

3. Results and discussion

The semipermeable surface (SPS) column selected for investigation in the present method can tolerate large numbers of small volume serum injections. Desilets et al. [17] and Pinkerton [18] have reviewed the construction of the stationary phase in the SPS column. Basically, the material has two phases. The outer phase is a porous, hydrophilic, polyoxyethylene polymer covalently bonded to the silica surface. The inner phase is hydrophobic, usually C18, C8, nitrile or phenyl. The serum protein recovery from a SPS column has been stated to be 97 ± 3% [18].

It is recommended by the manufacturer of the SPS column that the mobile phase composition should contain less than 25% organic modifier, have a pH in the 5.5–7.5 range, and an aqueous buffer concentration of 0.05–0.1 M [19]. These recommendations should aid in the prevention of protein deposition and pressure buildup in the HPLC system. These restrictions can make it difficult to separate closely related analytes such as phenylbutazone and oxyphenylbutazone in the presence of endogenous substances. Methanol, acetonitrile and isopropanol were investigated as organic modifiers for this assay. Acetonitrile was found to give the best peak height response with shorter retention times compared to methanol or isopropanol. Without acetonitrile in the mobile phase, the total run time of both analytes would

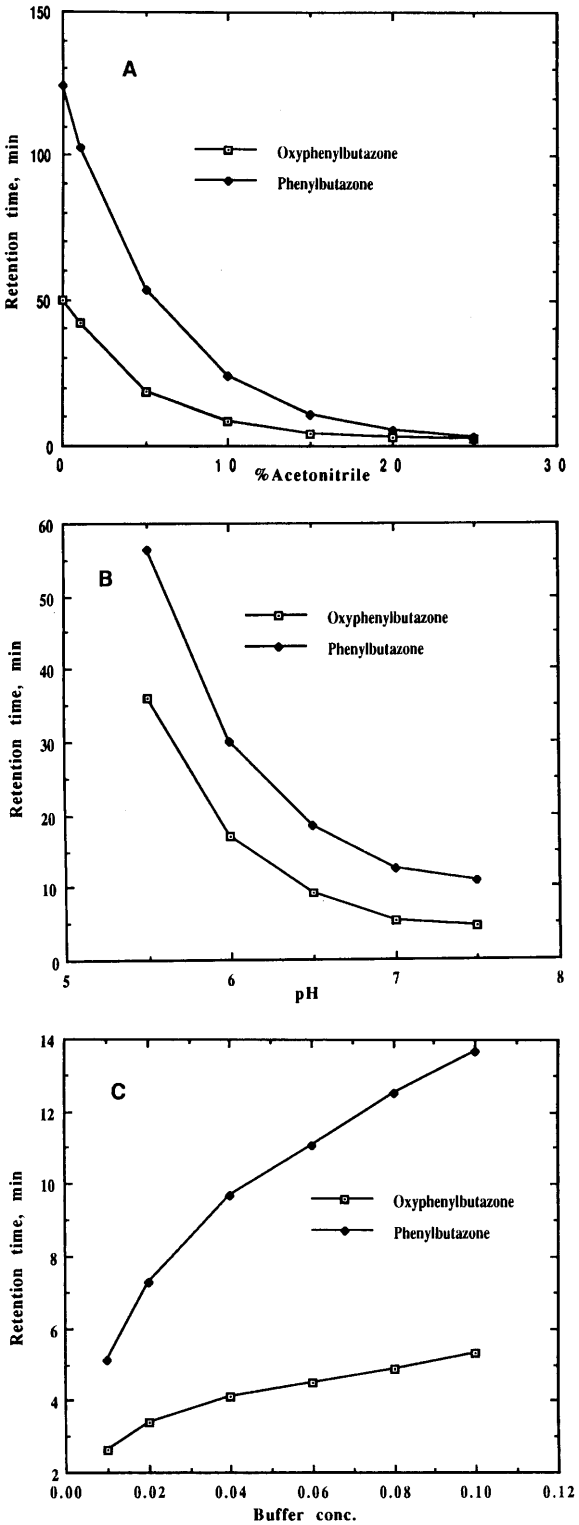

A
Oxyphenylbutazone
Phenylbutazone
Retention time, min
%Acetonitrile
B
Oxyphenylbutazone
Phenylbutazone
Retention time, min
pH
C
Oxyphenylbutazone
Phenylbutazone
Retention time, min
Buffer conc.

Fig. 2.

have been more than 2 h. As expected, maximum peak height response of each analyte was obtained at 25% acetonitrile in the mobile phase, but the retention times were too short (Fig. 2A) and analyte peaks were overlapped by endogenous serum peaks. Reduction of the acetonitrile concentration to 15% allowed the adequate separation of the analytes from any interfering endogenous peaks.

In this study, it was observed that pH not only affected retention time, but also peak height response. The response was much higher at basic vs acid pH, but at acidic pH, retention times were longer (Fig. 2B). Within the recommended 5.5–7.5 pH range, only a mobile phase of pH 7.5 allowed complete separation of analytes from endogenous interferences.

Phosphate buffer concentration in the mobile phase also affected retention time and peak height response. The buffer concentration was varied from 0.01–0.1 M and it was found that a 0.01 M buffer concentration gave the highest peak height response and shortest retention time (Fig. 2C). However, the final buffer concentration was set at 0.05 M since there was less observed build up of pressure in the HPLC system and more analyte retention. The final mobile phase composition for phenylbutazone and oxyphenylbutazone on the SPS column was 15:85 v/v acetonitrile-0.05 M phosphate buffer pH 7.5. Fig. 3 represents a typical chromatogram of phenylbutazone and oxyphenylbutazone in serum directly injected onto the SPS column. Separation of the analytes was achieved within 13 min with detection at 265 nm.

The Pinkerton or internal surface reversed phase (ISRP) column was compared to the SPS column in this lab since it was also designed for direct injection of drugs in serum. The ISRP column also consists of two phases [18]. The outer phase is a hydrophilic diol-glycine layer covalently

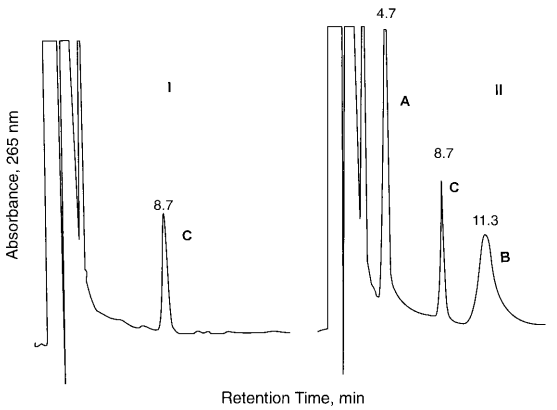


Fig. 3. Typical HPLC chromatograms of (I) blank serum and (II) spiked serum containing 2 μg ml^{-1} each of (A) oxyphenylbutazone and (B) phenylbutazone. Peak C is an endogenous serum component. HPLC conditions: SPS column; mobile phase was 15:85 v/v acetonitrile - 0.05 M phosphate buffer pH 7.5; flow rate − 1 ml min^{-1}; detection at 265 nm, injection volume − 20 μl, ambient temperature (23°C).

bonded to the silica surface. The inner phase is hydrophobic consisting of a diol-tripeptide layer. Retention of phenylbutazone and oxyphenylbutazone on the ISRP column, like the SPS column, was significantly influenced by organic modifier, pH and buffer concentration. In general, the SPS column was found to be more retentive of the analytes than the ISRP column within a limited study of acetonitrile concentration (2.5–20%), pH (6–7.5) and buffer concentration (0.05–0.2 M). The best separation of phenylbutazone and oxyphenylbutazone on the ISRP column was achieved using a mobile phase of 2.5: 97.5 v/v acetonitrile-0.2 M phosphate buffer pH 7. Retention times were 6.3 min for oxyphenylbutazone and 10.5 min for phenylbutazone (see Fig. 4).

Calibration curves were prepared on the SPS column for phenylbutazone and oxyphenylbutazone in serum at 0.5–20 μg ml^{-1} levels. Linear

Fig. 2. (A) Effect of acetonitrile concentration on retention time of oxyphenylbutazone and phenylbutazone in SPS column; mobile phase was acetonitrile-0.05 M aqueous phosphate buffer pH 7.5. (B) Effect of mobile phase pH on retention time of oxyphenylbutazone and phenylbutazone in SPS column; mobile phase was 15:85 v/v acetonitrile-0.05 M aqueous phosphate buffer. (C) Effect of buffer concentration on retention time of oxphenylbutazone and phenylbutazone in SPS column; mobile phase was 15:85 v/v acetonitrile-aqueous phosphate buffer pH 7.5. The separations were performed at a flow rate of 1 ml min^{-1}, detection at 265 nm and at ambient temperature (23°C).

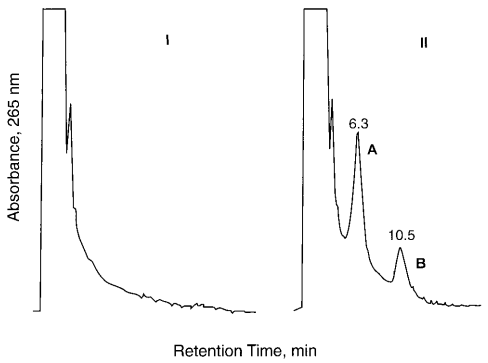


Fig. 4. Typical HPLC chromatograms of (I) blank serum and (II) spiked serum containing 5 μg ml^{-1} each of (A) oxyphenylbutazone and (B) phenylbutazone. HPLC conditions: ISRP column, mobile phase − 2.5: 97.5 v/v acetonitrile-aqueous 0.2 M phosphate buffer pH 7, flow rate − 1 ml min^{-1}, detection − 265 nm, injection volume − 20 μl, ambient temperature (23°C).

regression analysis for each analyte was performed using concentration vs peak height response. Typical correlation coefficient, slope and intercept for phenylbutazone were 0.999, 904 and − 256, respectively ($n = 6$) and for oxyphenylbutazone, 0.999, 3065 and 411, respectively ($n = 6$). An internal standard was unnecessary in this assay since there were no sample extraction steps. Accuracy and precision data for the direct injection method using spiked drug and metabolite samples are shown in Table 1. Intraday and interday reproducibility data for both analytes is shown in Table 2.

Recoveries of phenylbutazone and oxyphenylbutazone on the SPS column were determined to be 79.7 ± 2.7 and 94.2 ± 4.5%, respectively, comparing peak height response of known analytes concentrations in serum vs. buffer solution. It has been reported in the literature that total analyte concentration can be generally obtained on the SPS column at low μg ml^{-1} levels with ≤ 20 μl injections [19]. Injection volumes ≥ 350 μl have been used to give a measure of both free and bound analyte [20].

The injection volume of serum is usually restricted to 20 μl to enhance SPS column life. There was a noticeable pressure build-up over time in our HPLC system due to fouling of the frits despite the use of a guard column and filtered (0.22 μm) serum sample. Approximately 100 total injections could be made before the system pressure reached 4000 psi. Since it may not always be easy or convenient to remove column frits, future SPS column construction with easy removal and replacement of frits may alleviate the problem. Wong et al. [21] have suggested the use of a PTFE frit rather than stainless steel. The retention times of oxyphenylbutazone and phenylbutazone analytes decreased by 1 and 2.5 min, respectively, on the SPS column over a period of 4 months of direct injections.

4. Conclusions

The direct injection method for phenylbutazone and oxyphenylbutazone in serum using the SPS column is easy to perform, uses small sample volumes, requires no sample pretreatment steps and possesses the necessary sensitivity and reproducibility to be of use in pharmacokinetic and

Table 1
Accuracy and precision of oxyphenylbutazone and phenylbutazone added to serum

Analyte	Concn. added (μg ml^{-1})	Concn. found (μg ml^{-1})[a]	% Error	R.S.D.%
Oxyphenylbutazone	1.0	0.99 ± 0.05	1.0	5.1
	8.0	7.90 ± 0.06	1.3	0.8
	16.0	15.58 ± 0.17	2.6	1.1
Phenylbutazone	1.0	1.06 ± 0.05	6.0	4.7
	8.0	7.88 ± 0.09	1.5	1.1
	16.0	15.74 ± 0.14	1.6	0.9

[a] Mean ± S.D. based on $n = 3$.

Table 2
Intraday and interday accuracy and precision data

Concn. added (μg ml^{-1})	Oxyphenylbutazone			Phenylbutazone		
	Concn. found (μg ml^{-1})	% Error	R.D.S. (%)	Concn. found	% error	R.S.D. (%)
Intraday						
0.5	0.49 ± 0.01[a]	2.00	2.00	0.56 ± 0.02	12.0	3.60
1	0.95 ± 0.02	5.00	2.10	0.96 ± 0.04	4.00	4.17
2.5	2.45 ± 0.15	2.00	6.12	2.51 ± 0.08	0.40	3.19
5	5.04 ± 0.12	0.80	2.38	4.99 ± 0.22	0.20	4.41
10	10.07 ± 0.11	0.70	1.10	9.95 ± 0.30	0.50	3.02
20	19.98 ± 0.09	0.10	0.45	20.04 ± 0.14	0.20	0.70
Interday						
0.5	0.48 ± 0.04[b]	4.00	8.33	0.54 ± 0.04	8.00	7.40
1	0.97 ± 0.06	3.00	6.19	0.96 ± 0.04	4.00	4.17
2.5	2.51 ± 0.01	0.40	0.40	2.56 ± 0.10	2.40	3.90
5	5.14 ± 0.27	2.80	5.25	5.14 ± 0.26	2.80	5.10
10	9.77 ± 0.29	2.30	2.97	9.71 ± 0.25	2.90	2.57
20	20.06 ± 0.09	0.30	0.45	20.1 ± 0.07	0.50	0.35

[a] Mean ± S.D. based on $n = 3$.
[b] Mean ± S.D. based on $n = 9$; samples were assayed over 3 days.

toxicokinetic studies of both analytes. Unfortunately, the SPS column is not as valuable as its advertisement indicates due to significant pressure buildup in the HPLC system over 100 direct injections. Laboratories running 1000–10 000 samples per month may find the use of the SPS column to be too expensive and wasteful with unnecessary down time for cleaning of column frits or changing the guard column. Improvements in column performance are definitely needed before these columns will receive widespread attention and use.

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