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A supercritical fluid chromatographic method using packed columns for phenylbutazone and oxyphenbutazone in serum, and for phenylbutazone in a dosage form

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Abstract: The separation of phenylbutazone (PB) and its major metabolite oxyphenbutazone (OPB) using supercritical fluid chromatography (SFC) has been investigated. The separations were studied on octadecylsilane, silica and cyano packed columns with 5% methanol in carbon dioxide as mobile phase and detection at 240 nm. The octadecylsilane column showed the most favourable chromatographic parameters for the analysis of the analytes. Recoveries of PB and OPB from spiked human serum were in the 82–83% range using solid phase extraction on an ODS cartridge. Limits of detection of the SFC assay were 0.1 $\mu g\ ml^{-1}$ for PB and 1.0 $\mu g\ ml^{-1}$ for OPB. Accuracy and precision of the method were in the 0.24–4.94% range for PB and OPB. The SFC method was directly comparable to an HPLC assay of the same analytes. The SFC method was also applied to a commercial 100 mg dosage form of PB with good recovery of PB.

Keywords: *Supercritical fluid chromatography; phenylbutazone; oxyphenbutazone; packed columns; serum; dosage form.*

Introduction

Supercritical fluid chromatography (SFC) may be readily used in order to analyse drugs found in dosage forms and biological fluids. SFC has a definite advantage over GC in that polar drugs do not have to be derivatized for volatilization because of the high solvating capability of a supercritical fluid. SFC, unlike HPLC has the advantage of dramatically changing the chromatographic behaviour of an analyte without changing the composition of the mobile phase, and also no solvent disposal is required. The higher diffusivity of supercritical mobile phase, compared to liquids, results in higher chromatographic efficiency in SFC compared to HPLC. SFC does not have the sensitivity of GC or HPLC, but there is growing interest in the development of SFC assays using capillary and packed columns for the detection of drugs.

SFC work previously performed in our laboratory included the analysis of selected oestrogens [1], non-steroidal anti-inflammatory agents [2], and chlordiazepoxide, diazepam and their related compounds [3, 4] on capillary SFC columns with FID detection. These studies showed good separation with high efficiencies, but sensitivities were lacking due to injection size and column capacity. It was of interest to investigate the use of packed column SFC to increase sensitivity and maintain baseline separation. Phenylbutazone (PB) and its metabolite oxyphenbutazone (OPB) were selected as model compounds to investigate the use of packed column SFC in the analysis of these drugs in serum. The chemical structures if the analytes are shown in Fig. 1. A commercial capsule dosage form of PB was also studied. Previous literature describing the SFC analysis of PB includes a report by Crowther and Henion on the SFC/MS determination of PB and several unidentified hydroxylated metabolites in a crude equine urine acid extract [5]. The qualitative method was rapid (less than 5 min) and no chemical derivatization was necessary. The separation was achieved on a diol column using 10% methanol in carbon dioxide as mobile phase.

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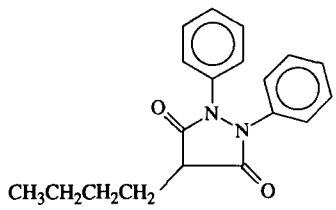
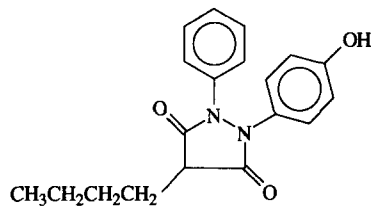


PHENYLBUTAZONE



OXYPHENBUTAZONE

Figure 1
Structures of phenylbutazone and oxyphenbutazone.

In this paper, an SFC assay for PB and OPB in a human serum sample and a PB capsule dosage form is reported. The procedure showed limits of detection in the 0.1 μg ml^{-1} level for PB and 1.0 μg ml^{-1} for OPB on a packed octadecylsilane (ODS) column using 5% methanol in carbon dioxide and UV detection at 240 nm.

Experimental

Reagents and chemicals

HPLC grade absolute methanol was purchased from J.T. Baker (Phillipsburg, NJ, USA). Supercritical grade carbon dioxide with 5% methanol was obtained from Scott Speciality Gases (Plumsteadville, PA, USA). Deionized-distilled water was obtained by a filtration system from Continental Water (Roswell, GA, USA). Solid phase extraction cartridges (ODS) were obtained from Varian Sample Preparation Products (Harbor City, CA, USA). Drug free human serum was obtained from Instrumentation Laboratory, Fisher Scientific Company (Cat no. 2906-34, Orangeburg, NY, USA).

Phenylbutazone and oxyphenbutazone were purchased from the Sigma Chemical Co. (St Louis, MO, USA). The commercial dosage form of phenylbutazone was obtained from a local pharmacy as 100 mg capsules distributed by Rugby (Rockville Centre, NY, USA).

Instrumentation

Chromatography was performed on a Lee Scientific Model 600D supercritical fluid chromatograph (Salt Lake City, UT, USA) equipped with a pump, oven, and UV/vis detector equipped with a Lee Scientific packed column flow cell assembly. This system was controlled by a Dell Computer (ACI 600D, software version 2.2). SFC was performed on three packed columns including; a 250 × 1 mm Deltabond 5-μm ODS, a 250 × 1 mm Deltabond 5-μm Cyano, and a 250 × 1 mm 5-μm Silica-60 purchased from Keystone Scientific Inc. (Bellefonte, PA, USA). An Eppendorf Centrifuge Model 5413 was obtained from Brinkman Instruments Inc. (Westbury, NY, USA).

Preparation of standard solutions

A 1 mg ml^{-1} stock solution (A) and a 0.1 mg ml^{-1} stock solution (B) each containing both PB and OPB were prepared in absolute methanol to be used in the preparation of the serum calibration curve. An additional standard solution (C) containing 40 μg ml^{-1} of PB in absolute methanol was prepared for use in the capsule assay.

Chromatographic conditions

A Deltabond 5 μm ODS, 250 mm × 1 mm i.d. column was utilized, with a fused silica tube of approximately 3.2 cm long and 10 μm i.d. × 150 μm OD attached to the outlet of the detector. The end of the restrictor was placed in a beaker containing absolute methanol. The time split was set at 10 s; 1 μl injection and UV/vis detector set at 240 nm was used. Supercritical fluid grade carbon dioxide with 5% methanol, was used as the mobile phase. The analysis time was 10–12 min, the pump pressure, 170 atm and the oven temperature was kept at 85–90°C.

Analysis of serum samples

To a 1 ml human serum sample contained in a 1-ml conical polypropylene vial was added 400 μl of 5% aqueous trichloroacetic acid. The vial was shaken vigorously for 20 s followed by centrifugation for 10 min at 11,500 rpm. The supernatant was transferred to a 1-ml glass volumetric tube. The plastic vial was washed once with 250 μl of water and vortexed for 30 s followed by centrifugation for 10 min at 11,500 rpm. The washing was transferred to the 1-ml volumetric tube. An ODS solid phase extrac-

tion (SPE) cartridge was conditioned with 2 ml of absolute methanol followed by 2 ml of water. The entire contents of the volumetric tube were added to the cartridge, and a vacuum was applied to draw the sample through the matrix. Then 500 μl of water was used to wash the volumetric tube and the washing applied to the cartridge. The cartridge was washed with 1 ml of water and the vacuum applied for 2 min. The PB and OPB analytes were eluted from the cartridge using eight 125-μl portions of absolute methanol. The eluent was collected in a 1-ml glass volumetric tube and absolute methanol added to volume. A 1-μl aliquot was injected into the SFC chromatograph.

Standard samples of PB and OPB for a serum calibration curve were prepared by accurately measuring 50 and 250 μl of stock solution B, and 100 μl of stock solution A into individual 1-ml conical plastic vials. The contents of each vial were evaporated to dryness under a nitrogen stream at ambient temperature. Then 1 ml of drug free human serum was added to each vial and the residue redissolved by manual inversion of each vial for 2 min to obtain final concentrations of PB and OPB in each sample of 5.0, 25.0 and 100.0 μg ml^{-1}, respectively. Treatment with trichloroacetic acid was performed as described above and the sample applied to the ODS cartridge. A blank consisting of drug-free human serum was also performed.

Analysis of phenylbutazone capsule

The contents of a 100 mg PB capsule was emptied into a 100-ml volumetric flask. A volume of 50 ml of absolute methanol was added, and the solution was sonicated for 10 min. The solution was vacuum filtered into a Buchner flask and transferred to a clean 100-ml volumetric flask with the aid of two 5-ml washings of absolute methanol. Absolute methanol was added to volume and 1:50 dilution was made to obtain a final concentration of 20 μg ml^{-1}. One microlitre of this PB solution was injected into the SFC chromatograph, and the peak height was compared to a calibration curve ranging from 10–40 μg ml^{-1} of PB prepared from standard solution C.

Results and Discussion

Three packed columns, silica, cyano and ODS, were tested to study the resolution of PB and OPB. In order to meet our preferred criteria for separation, both drugs had to elute between 5 and 15 min to avoid interference with the solvent front and minimize band broadening, respectively. It was also desirable for the calculated resolution value (*Rs*) of the two peaks to be between 1 and 2.

In SFC, three parameters (percentage of organic modifier in carbon dioxide, pressure on mobile phase and column oven temperature) can be changed independent of the packed column. Various amounts of methanol can be added to the carbon dioxide mobile phase to increase the polarity. Our particular chromatograph was not equipped with a mixer that would have allowed the percentage of organic modifier in the mobile phase to be varied; therefore, a commercial tank containing a premixed percentage of methanol in carbon dioxide was purchased. The pressure on the mobile phase can be also increased or decreased, within a range of 100–415 atm and the oven temperature can be varied within a range of 20–450°C.

The initial column investigated for the separation of PB and OPB was a silica column. When the temperature was set at 80°C with a pressure of 350 atm, both the PB and OPB eluted >10 min with large tailing factors and low column efficiencies as evidenced by band broadening. After more experimentation, the temperature was set at 50°C with a pressure of 250 atm. The chromatogram obtained under these conditions showed a PB peak at 3.18 min, which was extremely close to the solvent front (2 min). Oxyphenbutazone eluted at 10.12 min with a high column efficiency, but with a large tailing factor. A SFC chromatogram of the separation is shown in Fig. 2(a). The tailing noticeable with peak 2 is an artifact of the data acquisition system. With PB elution close to the solvent front and unfavourable tailing observed with OPB, other columns were investigated to overcome these disadvantages.

The cyano column showed weaker retentive interactions compared to silica (density of mobile phase of 0.716 g ml^{-1} compared to 0.864 g ml^{-1} on silica). A pressure of 300 atm and a 60°C oven temperature caused both drugs to elute near the solvent front. The temperature was raised to 70°C and the pressure lowered to 200 atm after various trials. These conditions gave retention times of 4.98 min for PB and 12.30 min for OPB. The

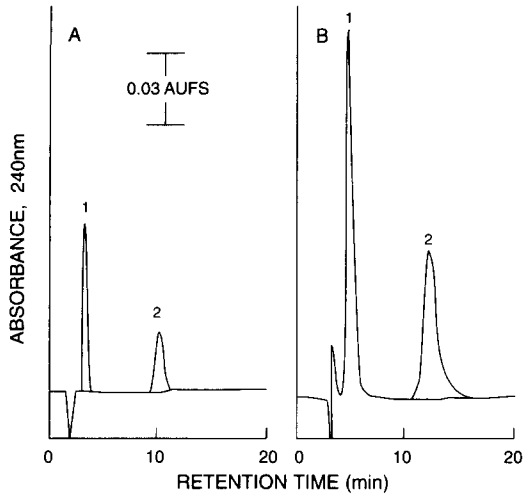


Figure 2
Typical SFC separations of 50 μg ml^{-1} PB and 50 μg ml^{-1} OPB on (a) silica column and (b) cyano column. SFC conditions: silica, 50°C, 250 atm, UV detection at 240 nm, cyano, 70°C, 200 atm, UV detection at 240 nm.

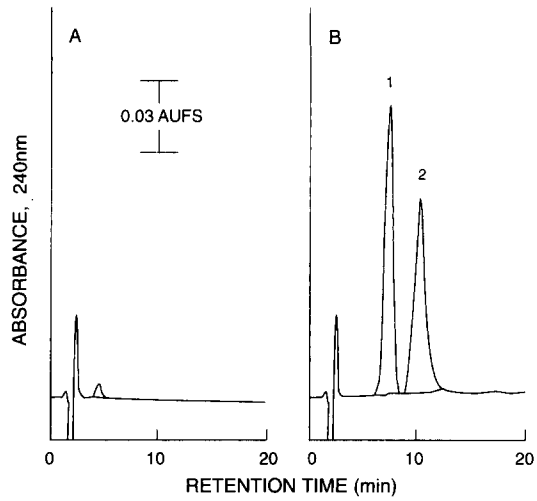


Figure 3
Typical SFC separation of PB and OPB on ODS column following solid phase extraction from serum sample. (a) Serum blank and (b) serum extract of PB (1) and OPB (2) at 50 μg ml^{-1}. SFC conditions: 86°C, 170 atm, UV detection at 240 nm.

PB peak again eluted too close to the solvent front (3.2 min). The cyano column efficiencies for both PB and OPB were lower than that observed on the silica column, but there was less tailing. A SFC chromatogram of the separation is shown in Fig. 2(b). There are no other components coeluting with peak 2. The shape is an artifact of the data acquisition system.

The final column studied was an ODS column. It showed a strong ability to retain PB, in particular, even when conditions were set to elute OPB around 10 min. Various temperatures and pressures were used, but it was found that the calculated resolution value for both compounds on the ODS column was smaller than that observed on the silica and cyano columns. With the temperature and pressure set at 86°C and 170 atm, respectively, separation of both drugs was achieved within 10 min with retention times of 7.05 and 9.97 min for PB and OPB, respectively (see Fig. 3b). These settings indicated that the ODS column provided less binding interaction for PB and OPB (mobile phase density of 0.528 g ml^{-1}) than was previously observed with the silica and cyano columns, thus allowing the drugs of interest to be suitably resolved (*Rs* of 1.57). This desirable resolution of PB and OPB was not obtainable on the silica and cyano columns, where resolution values of 5.71 and 3.96, respectively, were obtained. In addition, increased band broadening and/or tailing caused by OPB eluting >10 min and interference of PB with the solvent front were overcome. The ODS column gave a chromatogram showing a small amount of tailing of the PB and OPB peaks relative to the cyano and silica columns. The column efficiency of PB was highest on the ODS column, while OPB showed highest efficiency on the silica column. Peak tailing and a large retention time span between the elution of the analytes were the main disadvantages which led to the choice of the ODS column over the silica column. It would appear that programming conditions could be used to eliminate retention and/or peak tailing problems. However, temperature programming cannot be used with packed columns to give consistent results. Pressure programming could possibly have helped with the retention of OPB on silica or cyano, but not for PB. The resolution of PB and OPB on the ODS column was not sufficient to allow pressure programming to be applied to the separation. Analytical figures of merit for PB and OPB on the various packed columns studied are listed in Table 1.

The recoveries of PB and OPB from human serum were 82.0 ± 4.7 and 83.4 ± 5.2% (n = 5), respectively, based on 50 μg ml^{-1} samples of both analytes. The recoveries were calculated by comparing peak heights of extracted analytes to peak heights of the unextracted analytes.

Table 1
Analytical figures of merit for phenylbutazone and oxyphenbutazone on selected packed columns

Column	Analyte	*Rs*	T_f*	*N*†	k^1	R_t (min)	α
Silica	PB	5.71	1.40	388	0.49	3.18	7.67
	OPB		1.57	716	3.73	10.12	
Cyano	PB	3.96	1.26	369	0.55	4.98	5.13
	OPB		1.20	332	2.82	12.30	
ODS	PB	1.57	1.01	410	2.32	7.05	1.62
	OPB		1.15	409	3.30	9.97	

*Calculated at 5% peak height.
†Calculated as $n = 5.54\ (tr/W0.5)^2$.

Table 2
Comparison of SFC vs HPLC assays of PB and OPB in spiked serum samples on packed ODS columns

		SFC*			HPLC†		
Analyte	Concn added μg ml^{-1}	Concn found μg ml^{-1}	Percentage error	RSD (%)	Concn found μg ml^{-1}	Percentage error	RSD (%)
PB	12	11.4 ± 0.14‡	4.67	1.2	12.4 ± 0.01	3.08	0.08
	50	48.3 ± 0.29	3.40	0.6	50.4 ± 0.07	0.86	0.14
OPB	12	11.7 ± 0.43	2.75	3.7	12.4 ± 0.05	3.58	0.42
	50	47.6 ± 1.08	4.89	2.3	50.6 ± 0.06	1.16	0.13

*See chromatographic conditions for ODS column. Experimental Section, this paper.
†Mobile phase of 65:35 v/v methanol–aqueous 2% glacial acetic acid on a Phenomenex μ-Bondapak 10C18 (300 × 3.9 mm i.d.) column at 2 ml min^{-1} with detection at 240 nm, see [6].
‡Mean ± standard deviation based on $n = 3$.

Since an internal standard could not be found which would give good peak shape and suitable retention time, the external standard method was used for quantitation of both PB and OPB. Samples and standards were alternated during the injection step to assure the highest assay precision. Linear calibration plots of PB and OPB were generated by least-squares regression of the analyte peak height vs the respective analyte concentration in spiked drug-free human serum. The slope, intercept and correlation coefficient data for PB and OPB were 3008, 350 and 0.9994 ($n = 4$) and 1798, 584 and 0.9991 ($n = 4$), respectively.

The limits of detection and quantitation for PB and OPB were 0.10 μg ml^{-1} and 1.0 μg ml^{-1} in absolute methanol, respectively ($S/N = 3$). Since therapeutic levels of PB in serum are in the 50–150 μg ml^{-1} range, there should be no problem with the detection or quantitation of PB or OPB using this SFC method. The percentage error (accuracy) and precision of this assay were determined using spiked serum concentrations of PB and OPB. The data in Table 2 demonstrates the results obtained from these spiked samples. Figure 3(a and b) shows the chromatograms obtained for a serum blank and a spiked serum sample. Reproducibility of retention time for PB and OPB on the ODS column as measured by % RSD was 1.02 and 1.31%, respectively. There was no interference from endogenous components present in the serum blank. Any distortion in the peak shapes is an artifact of the data acquisition system. This supercritical fluid technique is shown to have merit in the analysis of PB and OPB in serum as demonstrated with accuracy and precision in the 0.24–4.94% range. Table 2 also shows a comparison of the SFC data to that received from an HPLC assay of PB and OPB in human serum. Intra-day and inter-day precision of the SFC assay for PB and OPB were in the 0.4–1.3% ($n = 6$) and 5–6% ($n = 18$) ranges, respectively. These good reproducibilities were obtained by pressurizing the syringe pump containing the methanolic carbon dioxide to 400 atms for 10 min prior to initiation of sample injections. The SFC method was also applied to a commercial 100 mg capsule dosage form containing PB. The assay showed that PB was present in

the capsule at 99.2 ± 0.93% (n = 3) of the labelled amount, clearly within the USP XXII requirements [7].

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