biological liquids. It is possible to perform 14 assays in ~3 hr including reagent blanks, standards, and controls. Thus, our method should be suitable both for research in pharmacology and in a chemistry laboratory.

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Determination of Azobenzene and Hydrazobenzene in Phenylbutazone and Sulfinpyrazone Products by High-Performance Liquid Chromatography

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Abstract □ A high-performance liquid chromatographic method has been developed for the simultaneous determination of azobenzene and hydrazobenzene in phenylbutazone and sulfinpyrazone raw materials and formulations. The drug raw material or formulation is shaken with 1 *N* NaOH and *n*-hexane and centrifuged. The *n*-hexane layer is injected into a chromatograph equipped with a 10-μm cyano-amino bonded phase column. Azobenzene and hydrazobenzene are detected at 313 and 254 nm, respectively; the sensitivities are ~1 and 2 ppm, respectively, in the raw materials and formulations.

Keyphrases □ Azobenzene—determination in phenylbutazone and sulfinpyrazone products by high-performance liquid chromagraphy □ Hydrazobenzene—determination in phenylbutazone and sulfinpyrazone products by high-performance liquid chromatography □ High-performance liquid chromatography—determination of azobenzene and hydrazobenzene in phenylbutazone and sulfinpyrazone products

Hydrazobenzene is an intermediate in the manufacture of phenylbutazone and sulfinpyrazone (1). These drugs may be contaminated with hydrazobenzene as a result of incomplete clean up after manufacture or if the drugs degrade by hydrolytic ring opening and subsequent cleavage of the residual amido function (2). Azobenzene forms readily by autoxidation of hydrazobenzene (2); its presence in drugs may be due to the use of impure hydrazobenzene during manufacture or to the oxidation of hydrazobenzene (2). Recent work indicates that hydrazobenzene is a carcinogen in rats and mice (3) and that azobenzene is a carcinogen in rats, but not in mice (4).

There appear to be few available methods for the determination of azo- and hydrazobenzene. Hydrazobenzene (5) and azobenzene (6, 7) have been detected in phenylbutazone formulations by TLC, and a high-performance liquid chromatography (HPLC) method for the determination of hydrazobenzene in aqueous media (8) has been reported. An HPLC method for the simultaneous determination of azo- and hydrazobenzene in phenylbutazone and sulfinpyrazone drug raw materials and formulations is described here.

EXPERIMENTAL

Materials—Azobenzene[1], hydrazobenzene[2] (1,2-diphenylhydrazine), sodium hydroxide[3], HPLC-grade *n*-hexane[4], and absolute ethanol[5] were used as received. All solvents were flushed with nitrogen prior to use.

Apparatus—The liquid chromatograph consisted of a single-piston metering pump[6], an injector[7] equipped with a 100-μl sample loop, a dual-channel UV detector[8] (254 nm, 0.02 AUFS and 313 nm, 0.05 AUFS) and two 10-mV strip chart recorders[9] (chart speed of 0.5 cm/min). A Partisil-10 PAC[10] analytical column (25 cm × 4.6-mm i.d.) and a Porasil 400[11] (37–75 μm) precolumn (10 cm × 4 mm-i.d.) were used at ambient temperature with a mobile phase flow rate of 2 ml/min. The mobile phase was a solution of 2.5% absolute ethanol in *n*-hexane (v/v) flushed with nitrogen.

Calibration Curves—*Azobenzene*—A solution of azobenzene in

[1] Pfaltz and Bauer Inc., Stamford, Conn.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] Analytical Reagent Grade.
[4] Burdick and Jackson Laboratories, Muskegon, Mich.
[5] Consolidated Alcohols Ltd., Toronto, Ont.
[6] Model 110A; Altex Scientific, Berkeley, Calif.
[7] Model CV-6-UHPa-N_{60}; Valco Instruments Co., Houston, Tex.
[8] Model 440; Waters Associates, Milford, Mass.
[9] Linear Instruments Corp., Irvine, Calif.
[10] Whatman Inc., Clifton, N.J. (Cyano-amino polar phase bonded to silica gel).
[11] Waters Associates, Milford, Mass.

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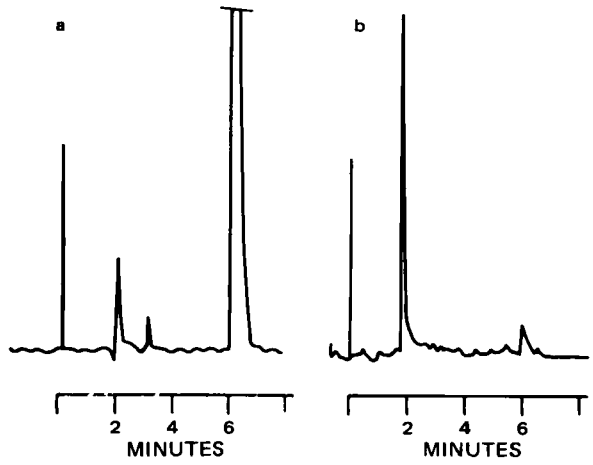

Figure 1—*Chromatograms of azobenzene (2.0 min) and hydrazobenzene (6.1 min). Key: (a) detection at 254 nm, (b) detection at 313 nm.*

n-hexane (1.6 μg/ml) was diluted with the same solvent to obtain five standard solutions ranging from 0.048 to 0.80 μg/ml. Duplicate 100-μl aliquots representing on-column amounts of 0.0048–0.080 μg of azobenzene were chromatographed at 313 nm with the signal attenuated to 0.05 AUFS.

Hydrazobenzene—A solution of hydrazobenzene in *n*-hexane (1.6 μg/ml) was diluted with the same solvent to obtain five standard solutions ranging from 0.10 to 1.6 μg/ml. Duplicate 100-μl aliquots representing on-column amounts of 0.010–0.160 μg of hydrazobenzene were chromatographed at 254 nm with the signal attenuated to 0.02 AUFS.

Daily Calibration—Two standard solutions of each compound (azo- and hydrazobenzene) were prepared fresh each day. The concentrations of the standards were such that the on-column levels were ~0.006 and 0.060 μg for azobenzene and ~0.016 and 0.120 μg for hydrazobenzene.

System Check Sample—Powdered, well-mixed composites were prepared from 50 capsules (or tablets) of phenylbutazone or sulfinpyrazone products which were known to contain azobenzene and hydrazobenzene. These were analyzed periodically to check the reproducibility of the chromatographic system and the sampling procedures.

Analysis of Phenylbutazone or Sulfinpyrazone Preparations—Phenylbutazone or sulfinpyrazone drug raw material (100 mg), or an aliquot of a composite of powdered tablets or capsule contents equivalent to 100 mg of the drug substance, was shaken with 10.0 ml of 1 *N* NaOH just long enough to wet the solid mass. To this was added 5.0 ml of *n*-hexane, and the mixture was shaken for 5 min on a horizontal shaker. After centrifuging at 4000 rpm for 1 min, the upper organic layer containing the azobenzene and/or hydrazobenzene was immediately separated from the aqueous phase containing the drug. Duplicate 100-μl aliquots of the organic phase were chromatographed with the 254- and 313-nm detectors in series. When the peak for the azobenzene and/or hydrazobenzene was off-scale, the sample was diluted with *n*-hexane and injected again. Quantitation was by the peak height technique for both impurities. Calibration standards of azobenzene and hydrazobenzene in *n*-hexane were used as the external reference solutions.

RESULTS AND DISCUSSION

During development of the method it was found that some sulfinpyrazone samples contained an impurity which was eluted at 1.8 min and was incompletely resolved from azobenzene at 2.0 min. This impurity was shown to be toluene by GC–MS. Toluene and azobenzene were resolved at retention times of 2.8 and 4.0 min, respectively, by elimination of ethanol from the mobile phase. This, however, delayed the elution of hydrazobenzene to such an extent that there was an unacceptable loss of sensitivity. Therefore, ethanol was retained in the mobile phase and the azobenzene was quantitated at 313 nm, where absorption by toluene is insignificant. The hydrazobenzene, which was eluted at 4.9 min, was quantitated by measurements at 254 nm. Typical chromatograms at both wavelengths are presented in Fig. 1.

Hydrazobenzene is reported to be oxidized readily in solvents such as benzene, methylene chloride, and methanol (8). In *n*-hexane in equilibrium with the atmosphere, hydrazobenzene was oxidized to azobenzene at a rate of ~5%/hr at room temperature. In nitrogen-flushed *n*-hexane there was no increase in the azobenzene level during the first 30 min and only a 4% conversion after ~2 hr. In addition it was found that azo- and hydrazobenzene in the nitrogen-flushed calibration solutions were stable over a period of 1 hr, as indicated by the constancy of the response factors of the five calibration solutions. Over the period of the extraction neither drug was degraded sufficiently to cause detectable increases in the azo- and hydrazobenzene levels. For phenylbutazone, the levels began to increase after 15 min, but no change was observed in sulfinpyrazone after 50 min.

The reproducibility of the procedure used for the extraction of azo- and hydrazobenzene from formulations was investigated using phenylbutazone and sulfinpyrazone formulations known to contain azo- and hydrazobenzene. Using 10 ml of 1 *N* NaOH and 5 ml of *n*-hexane as provided for by the method, the extraction of both contaminants was invariant between 50 and 250 mg of phenylbutazone and 50 and 150 mg of sulfinpyrazone. Increasing the amount of *n*-hexane used in the extraction from 5 to 10 and 5 to 20 ml for sulfinpyrazone and phenylbutazone, respectively, had no effect on the amounts of azo- and hydrazobenzene detected. The partition of azo- and hydrazobenzene into *n*-hexane from sodium hydroxide solution is virtually complete. This was demonstrated by shaking a solution of the contaminants dissolved in *n*-hexane with 1 *N* NaOH. There was no detectable change in the observed levels of azo- and hydrazobenzene after shaking with the base. Drug raw materials and formulations were spiked with known amounts of azo- and hydrazobenzene. Recoveries ranged between 85 and 110%.

The response of the chromatographic system was linear from 4 to 70 ng of azobenzene on-column, with an intercept that did not vary significantly from zero. The relative standard deviation of the ratio of the peak height to the weight of azobenzene was 2.3% for five calibration points. For hydrazobenzene the linear range was from 10 to 150 ng with a corresponding relative standard deviation of 3.5%. These ranges would correspond to azo- and hydrazobenzene levels of 2–35 ppm and 5–75 ppm, respectively, in 100 mg of drug when assayed by the procedure described in this paper. The chromatographic reproducibilities were established by injecting six aliquots of each of two solutions of azo- and hydrazobenzene. On-column amounts of 0.0057 and 0.032 μg of azobenzene gave relative standard deviations of the peak height–weight ratio of 2.34 and 0.53%, respectively. For hydrazobenzene, the relative standard deviations were 5.99 and 3.12% for on-column quantities of 0.0737 and 0.145 μg, respectively. The reproducibility of the assay for azo- and hydrazobenzene in formulations of phenylbutazone and sulfinpyrazone ranged between 4 and 13%, in general agreement with recoveries from the spiked samples.

The minimum detectable amounts of azobenzene and hydrazobenzene on column are ~1.5 and 4 ng, respectively. These levels corresponded to ~1 and 2 ppm of azobenzene and hydrazobenzene, respectively, for an on-column equivalent of 2000 μg of the drug. The minimum quantifiable levels were about twice these amounts.

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