Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5339

Note

Improved stability of phenylbutazone for its determination by liquid chromatography

RAM N. GUPTA

Department of Laboratory Medicine, St. Joseph's Hospital, Hamilton, Ontario L8N 4A6 (Canada)

(First received January 29th, 1990; revised manuscript received March 29th, 1990)

Phenylbutazone is still widely used as an anti-inflammatory agent in horses despite its serious hematologic side-effects. For the differential diagnosis of gastrointestinal bleeding in horses, the determination of the phenylbutazone concentration in the plasma of the animal is helpful to rule out the recent use of phenylbutazone by the animal. Liquid chromatography is the preferred thechnique for the determination of phenylbutazone in biological fluids because of its high UV absorbance, good chromatographic properties on reversed-phase columns and relatively high therapeutic range [1–5]. However, phenylbutazone is susceptible to extensive degradation during sample handling. In a recent paper Franssen *et al.* [5] have described a sample preparation procedure to minimize the degradation of phenylbutazone. However, this procedure gave variable recoveries of phenylbutazone. In this paper an alternative, relatively simple procedure for the extraction of plasma for the determination of phenylbutazone is described.

EXPERIMENTAL

Standards

Stock phenylbutazone (Sigma, St. Louis, MO, U.S.A.), 5 mg/ml, was prepared in methanol. This solution was stored in 0.5-ml aliquots at −20°C in tightly capped glass tubes. Working phenylbutazone, 1 mg/ml, was prepared by diluting the stock solution with 0.1 *M* ammonium hydroxide. Plasma standards of 0.5–80 mg/l were prepared by adding appropriate volumes of working phenylbutazone solution to aliquots of horse serum (Qualicum Scientific, Nepean, Canada). The standards were stored in 0.5-ml aliquots at −20°C.

Extraction

A 100 mm × 13 mm extraction tube was placed in a 150 mm × 25 mm test tube, and a 1-ml Extrelut column (EM Industries, Gibbstown, NJ, U.S.A.) was placed on the extraction tube. (The outer tube acted as a stand for the column.) A

0378-4347/90/$03.50

0.25-ml aliquot of 0.25 *M* monobasic sodium phosphate freshly spiked with 50 mg/ml sodium bisulfite was applied to the column followed by 0.25 ml of the sample and allowed to stand for 15 min. The column was eluted twice with 4-ml aliquots of pentane allowing the column to drain completely. The combined eluate was mixed with 0.25 ml of 0.5% tetramethylammonium hydroxide, prepared fresh by diluting 0.2 ml of a 25% methanolic solution of tetramethylammonium hydroxide (Sigma) to 10 ml with deionized water, for 10 min on a rotary mixer. The mixture was centrifuged for 5 min at ambient temperature at 800 *g* and a 5-μl aliquot of the lower aqueous phase was injected into the liquid chromatograph using a 10-μl syringe.

Chromatography

A modular chromatographic system comprising of a Model 100 A pump (Beckman Instruments, Berkeley, CA, U.S.A.), a 20-μl loop syringe loading injector (Model 7125, Rheodyne, Cotati, CA, U.S.A.), a 15 cm × 4.6 mm I.D. reversed-phase Ultrasphere ODS silica column (5-μm particles, Beckman) protected by an RP-18 1.5 cm × 4.6 mm I.D. guard cartridge (7-μm particles, Brownlee Labs., Santa Clara, CA, U.S.A.), a Model SPD-6AV absorbance detector (Shimadzu Scientific Instrument, Columbia, MD, U.S.A.) and a Model C-R3A integrator plotter (Shimadzu) was used. The chromatography was performed at ambient temperature. The mobile phase consisting of acetonitrile (550 ml)–water (450 ml)–70% perchloric acid (0.2 ml) was pumped at a flow-rate of 1.0 ml/min with an operation pressure of 7.8 MPa. The peaks were monitored at 240 nm.

RESULTS AND DISCUSSION

Fig. 1A shows a chromatogram of an extract of drug-free serum and Fig. 1B of an extract of serum spiked with 10 mg/l phenylbutazone. There are only few extraneous peaks. Pentane is a highly selective solvent and does not give any peak due to its poor absorbance of UV light. Fig. 1C shows a chromatogram of an extract of serum obtained from a 600-kg horse 8 h after the administration of an oral 1750-mg phenylbutazone dose.

The recovery of phenylbutazone as determined by comparing the peak areas of 5-μl injections of an extract of a 10 mg/l plasma standard and a 10 mg/l standard in 0.5% tetramethylammonium hydroxide is about 90%. The present extraction procedure is designed to exclude neutral and basic compounds. Further, a number of acidic compounds like oxyphenbutazone, barbiturates, hydantions, succinimides and sulfonylureas are also not extracted because of their poor solubility in pentane. The relationship between the peak areas and the concentration of phenylbutazone is linear for the range tested (0.5–80 mg/l) and the curve passes through the origin. Phenylbutazone standards prepared in human plasma behave similarly to standards prepared in horse serum with respect to extraction recovery and the presence of extraneous peaks.

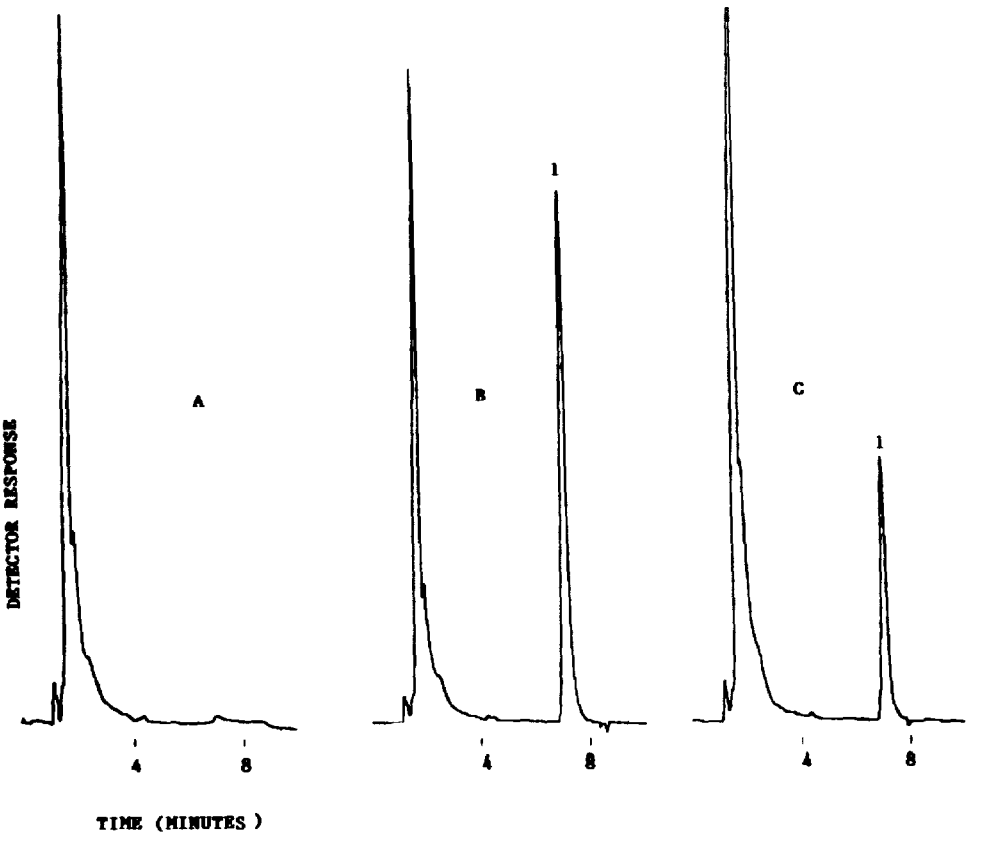

Fig. 1. Chromatograms of extracts of (A) drug-free horse serum, (B) 10 mg/l phenylbutazone standard in horse serum and (C) serum obtained from a 600-kg horse 8 h after the administration of a 1750-mg dose of phenylbutazone orally. Peak 1 = phenylbutazone. Detector output, 0.8 V; plotter attenuation, 3; chart speed, 0.5 cm/min.

I could not find a commercially available compound which was suitable to be used as an internal standard. Monophenylbutazone (Sigma), a structural analogue of phenylbutazone, elutes close to the solvent peak with the described mobile phase. The described extraction procedure is simple and allows precise quantitation of phenylbutazone without the use of any internal standard. Analysis of plasma supplemented with 20 mg/l phenylbutazone showed a within-batch relative standard deviation (R.S.D.) of 9.8% ($n = 10$, mean = 19.5 ml/l) and a between-batch R.S.D. of 12.4% ($n = 8$, mean = 19.2 mg/l) over a ten-week period. There was no improvement of R.S.D. when glyburide was added as an external standard to tetramethylammonium hydroxide reagent prior to back-extraction of pentane extract of plasma into aqueous phase. There is no evidence of any damage of the silica-based guard or analytical column even after 100 injections of 5-μl aliquots of 0.5% tetramethylammonium hydroxide solution. Injection of upto 10

μl of the alkaline solution does not affect the peak shape. However, injections of the alkaline extract higher than 10 μl tend to split the peak of phenylbutazone.

Phenylbutazone is quite stable at alkaline pH. There is no change in the area of phenylbutazone when the extract is left at room temperature for 48 h. Between-batch precision data indicate that plasma standards stored frozen at $-20°C$ are quite stable. Phenylbutazone plasma standards of 20 mg/l stored at 4°C for a week also did not show any change in the concentration of phenylbutazone. Methanolic solutions of phenylbutazone have been stored in small aliquots for four weeks at 4°C and for three months at $-20°C$ without any observable degradation of phenylbutazone. However, there is a gradual decomposition of phenylbutazone when aliquots of the solution are withdrawn from the same tube which is stored at 4°C or at $-20°C$ over a three-month period.

In conclusion, the described procedure allows the specific determination of phenylbutazone avoiding its degradation during sample preparation.

ACKNOWLEDGEMENTS

Thanks are due to Dr. M. Chernesky of St. Joseph's Hospital for providing horse blood after the administration of phenylbutazone dose. I thank Miss Fadia Ros for the preparation of this manuscript.

REFERENCES

1 M. Alvinerie, *J. Chromatogr.*, 181 (1980) 132.
2 T. Marunaka, T. Shibata, Y. Minami and Y. Umeno, *J. Chromatogr.*, 183 (1980) 331.
3 L. Aarons and C. Higham, *Clin. Chim. Acta*, 105 (1980) 377.
4 L. G. Tillman and G. E. Hardee, *Anal. Lett.*, 18 (1985) 1897.
5 M. J. A. M. Franssen, Y. Tan, I. Freij, C. A. M. van Ginneken and F. W. J. Gribnau, *Pharm. Weekbl. Sci. Ed.*, 8 (1986) 229.