Journal of Chromatography, 303 (1984) 222–224
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,129

Note

Effect of precipitating agents on the analysis of metronidazole by high-performance liquid chromatography

GERALD A. WOOLLARD
Department of Clinical Chemistry, Auckland Hospital, Park Road, Grafton, Auckland (New Zealand)
(Received August 6th, 1984)

Metronidazole [Flagyl; 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] is a drug used in the treatment of trichomonas, amoeboid and anaerobic injections[1].

Previous reports on the analysis of metronidazole by high-performance liquid chromatography (HPLC) have been published using extraction[2], dilution with mobile phase[3] and protein precipitation[4,5] for sample preparation. The drug lends itself to analysis following protein precipitation and therefore, for routine monitoring, for therapeutic purposes a method based on ref. 4 was adopted. The nature of the precipitating agent was found to grossly affect the quality of the chromatogram.

EXPERIMENTAL

Instrumental conditions

The column used was a 30 cm × 3.9 mm I.D. Waters C_{18} column (10 μm particle size) at a temperature of 35°C. The mobile phase was acetonitrile–0.1 *M* phosphate buffer, pH 4.0 (15:85) or methanol–0.1 *M* phosphate buffer, pH 4.0 (15:85) at a flow-rate of 2 ml/min. UV detection was at 325 nm, 0.08 a.u.f.s., chart speed 0.5 cm/min. The internal standard was 1-(3-chloro-2-hydroxypropyl)-2-nitroimidazole (Roche R07-0269), 30 mg/100 ml methanol.

Methods

A 0.3-ml volume of serum (sample or control) was placed into a centrifuge tube, and 0.5 ml of precipitant (from methanol, ethanol, isopropanol or acetonitrile) and 50 μl volume of internal standard were added. The mixture was vortexed briefly and allowed to stand for 10 min, and then centrifuged at 900 *g* for 5 min. A 50-μl volume of supernatant was injected.

RESULTS AND DISCUSSION

In the routine clinical analysis of drugs by HPLC the speed of analysis is an important consideration in addition to the other usual requirements such as sensitivity, selectivity, accuracy etc. The duration of most chromatograms is relatively short but this can be offset by extensive sample cleanup. Of the most commonly used sample preparation schemes, protein precipitation followed by direct injection of the

0021-9673/84/$03.00

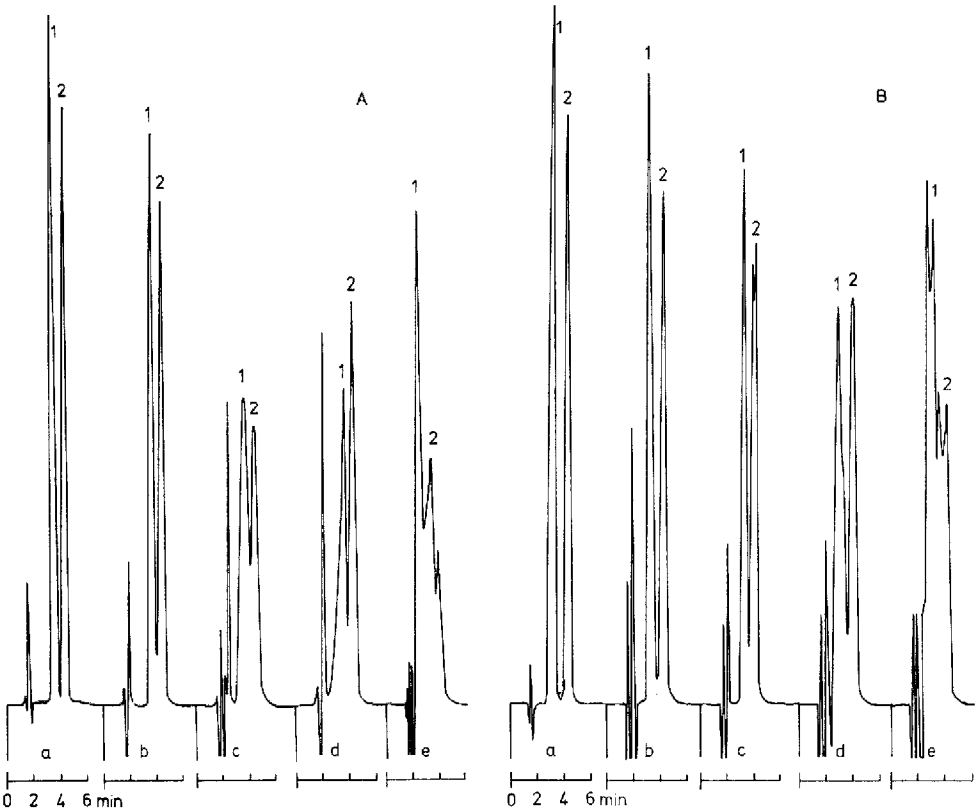

Fig. 1. Effect of precipitant on the peak shapes of metronidazole and the internal standard. (A) Mobile phase, acetonitrile–phosphate buffer (15:85); (B) mobile phase, methanol–phosphate buffer (15:85). Peaks: 1 = metronidazole, 2 = internal standard. Solvents: a = mobile phase, b = methanol–water (62:38); c = ethanol–water (62:38); d = acetonitrile–water (62:38); e = isopropanol–water (62:38).

supernatant is clearly the fastest and should be considered whenever possible.

The most commonly used solvents for precipitation of serum proteins are the water miscible alcohols and acetonitrile. Each of these were investigated to test their ability to produce a clear interference free supernatant solution for metronidazole analysis. It became apparent that the peak shape of the metronidazole and internal standard varied grossly depending on which precipitating solvent was used. Fig. 1 shows this for mobile phases consisting of both aqueous methanol and aqueous acetonitrile. The chromatograms given are those of standards dissolved in a 62% mixture of the precipitant with water which is equivalent to the proportions expected by following the stated method. Higher proportions of precipitant gave greater peak destruction. The same behaviour was observed with a new or well-used column. Only methanol gave a usable chromatogram. It is interesting to note that the loss of peak shape is different to the characteristic broadening and tailing seen with the loss of column efficiency. The solvent interference involves mainly peak broadening, peak splitting and skewing of the peak forwards. It occurs in an unpredictable manner which is not strictly reproducible (Fig. 1 gives typical patterns). Marques *et al.*[5], who use the same column, precipitate the serum proteins with an equal volume of ethanol

but do not report the same problems. They use a lower concentration of acetonitrile in the mobile phase in order to detect the 2-hydroxymethyl metabolite of metronidazole. This may have an influence.

The efficiency of various precipitants in removing proteins has been studied by Blanchard[6]. Of the organic solvents examined in the report, methanol is the least effective, *i.e.* acetonitrile > acetone > ethanol > methanol. It is evidence from this that the loss of peak shape in the metronidazole analysis is not due to the presence of co-injected proteins. It is more likely to be a result of mobile phase disturbance. This fits the pattern that the peak shape gets worse as the alcohol gets more hydrophobic (Fig. 1).

The proportion of methanol used by us is 1.66 volumes (*i.e.* 0.5 ml) to 1 volume (*i.e.*, 0.3 ml) of sample resulting in a 62% methanol supernatant. According to the data reported by Blanchard[6] this will give 98% precipitation of the serum proteins. A smaller proportion of methanol, say 1 volume to 1 volume of sample, will only give 73% precipitation which, although a good peak shape will be achieved, is unacceptable in terms of column deterioration. A higher proportion such as the 3.3 volumes of methanol to 1 volume of sample used in ref. 4 results in a slight improvement in protein precipitation, 99% according to Blanchard[6], but in our experience causes an inferior peak shape.

It is apparent from this that in the analysis of metronidazole (and similar drugs) by HPLC, careful consideration should be given to the nature and proportions of the precipitating agent.

REFERENCES

1 *Anaerobicidal Agent Flagyl (Metronidazole)*, May and Baker, Dagenham, 1979.
2 A. Gulaid, G. W. Houghton, O. R. W. Lewellen, J. Smith and P. S. Thorne, *Br. J. Clin. Pharmacol.*, 6 (1978) 431.
3 L. A. Wheeler, M. De Meo, M. Halula, L. George and P. Heseltine, *Antimicrob. Agents Chemother.*, 13 (1978) 205.
4 *Bulletin E-3360*, Roche, Basle.
5 R. A. Marques, B. Stafford, N. Flynn and W. Sadee, *J. Chromatogr.*, 146 (1978) 163.
6 J. Blanchard, *J. Chromatogr.*, 226 (1981) 455.