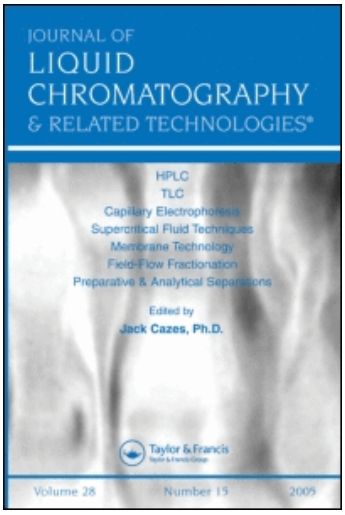

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Botsoglou, N. A. , Fletouris, D. J. , Psomas, I. E. and Mantis, A. I.(1997) 'Retention Profile of Fenbendazole and its Sulphoxide, Sulphone, and Hydroxylated Metabolites in Ion-Pair Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 20: 8, 1175 — 1184
To link to this Article: DOI: 10.1080/10826079708010967
URL: http://dx.doi.org/10.1080/10826079708010967

J. LIQ. CHROM. & REL. TECHNOL., 20(8), 1175–1184 (1997)

RETENTION PROFILE OF FENBENDAZOLE AND ITS SULPHOXIDE, SULPHONE, AND HYDROXYLATED METABOLITES IN ION-PAIR LIQUID CHROMATOGRAPHY

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ABSTRACT

The retention behavior of fenbendazole and its sulphoxide, sulphone, and p-hydroxylated metabolites as a function of the nature and the concentration of the ion-pairing reagent in the mobile phase has been investigated. The influence of negatively and/or positively charged pairing ions on peak shape has been discussed, whereas the effect of column temperature on retention has been evaluated.

INTRODUCTION

Fenbendazole, a benzimidazole drug, has become an integral part of the animal producing industry for control of internal worm parasites.[1] Despite the large scale use of this anthelmintic, adequate methods for the analysis of fenbendazole and its metabolites as residues in edible tissues and milk do not currently exist. Reported analytical methods,[2-4] although valuable for studying the routes of metabolism and excretion of fenbendazole in cattle,[5] goat,[6] and

rabbit[7] they may be. cannot offer the sensitivity required to ensure that animal derived human foods do not contain residues that exceed legal levels. The maximum residue limit of total fenbendazole residues in edible tissues and milk has been recently set at 10 ng/g.[8,9]

In this regard we have been developing new methods for the determination of fenbendazole residues in milk.[10] As a result of these early approaches, we have developed various ion pair liquid chromatographic systems to better control the selectivity and elute fenbendazole and its sulphoxide metabolite in a single run without tailing.[11] We report here a further examination of this ion-pair methodology for the efficient liquid chromatographic separation of fenbendazole and its sulphoxide, sulphone, and p-hydroxylated metabolites at trace residue levels.

EXPERIMENTAL

Instrumentation

Liquid chromatography was carried out with a Gilson system consisting of a Model 805 manometric module, a Model 305 piston pump, a Model 119 UV-vis detector, and a Model TC 831 column heater (Gilson Medical Electronics, Villiers-le-Bel, France). Injections were made on a Hichrom, 250x4.6 mm, stainless-steel column packed with Nucleosil 120 C_{18}, 5-μm, through a Rheodyne, Model 7725, injection valve (Cotati, CA) equipped with 100-μL sample loop. Recordings were made with a Kipp & Zonen, Model BD 111, pen recorder (Delft, Holland).

A Model D7402 EasyPure UV compact ultrapure water system (Barnstead/Thermolyne Corp., Dubuque, IA) was also used for preparing highly purified water.

Chemicals

Lichropur grade octanesulphonate sodium salt and tetrabutylammonium hydrogen sulfate, analytical grade phosphoric acid, and LC grade acetonitrile were from Merck (Darmstadt, Germany). Standard fenbendazole sulphoxide, fenbendazole sulphone, p-hydroxyfenbendazole, and fenbendazole (Fig. 1) were a gift from Hoechst Hellas (Athens, Greece).

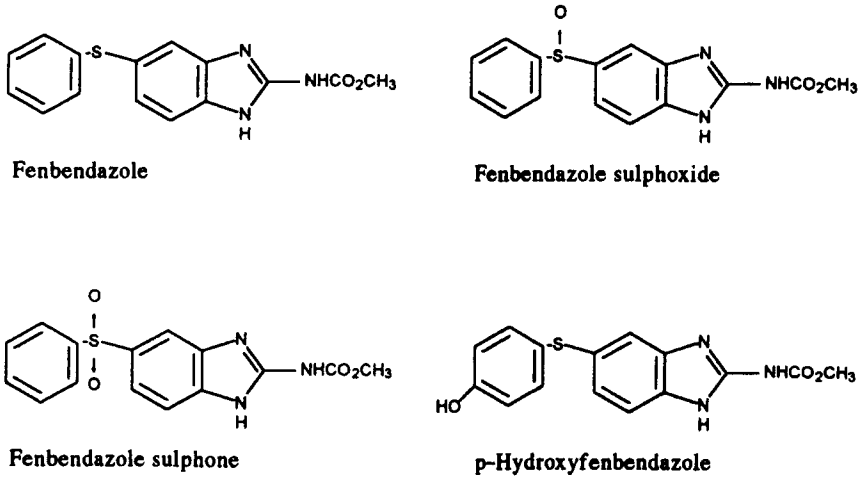

Figure 1. Chemical structures of investigated benzimidazoles.

Standard Solutions

Individual stock solutions of each reference standard were prepared in 10-mL volumetric flasks by weighing ca 5 mg of the compound and dissolving to volume with dimethylsulphoxide. A mixed standard intermediate solution containing all 4 analytes was prepared by combining appropriate aliquots (120-500 μL) from each of the stock solutions in a 10-mL volumetric flask and diluting to volume with acetonitrile. Mixed standard working solutions were prepared by diluting appropriate aliquots of the mixed intermediate solution in the mobile phase used each time.

Chromatographic Conditions

The mobile phases used in this study consisted of acetonitrile and 0.01 M phosphoric acid or phosphate buffer (pH 7). The acidic mobile phases differed from each other in both the type and the concentration of the contained ion-pair reagent. Some contained octanesulphonate sodium salt or tetrabutylammonium hydrogen sulfate, whereas others contained a mixture of these pairing ions at a molar ratio of 1/1 or 1/2. Following their preparation, each mobile phase was passed through 0.2 μm Nylon-66 filter (Anachem, Luton, UK) and degassed using helium. The mobile phase was delivered in the system at a rate of 1 mL/min.

The Nucleosil 120 C_{18} stationary phase was thoroughly equilibrated with mobile phase each time before use. Reproducible capacity factors (k') could be obtained after passage through the column of at least 70 mL of mobile phase. When the mobile phase contained ion-pair reagents, passage of 150-mL volume was indispensable for column equilibration. On changing the mobile phase, successive column washings with at least 100 mL portions of water and acetonitrile were quite indispensable for removing the adsorbed pairing ions. Detection was made at 290 nm at a sensitivity setting of 0.05 a.u.f.s. Chart speed was set at 5 mm/min.

RESULTS AND DISCUSSION

Separation of the studied analytes by literature reversed phase liquid chromatographic methods[2-4] was generally characterized by poor chromatographic performance. The highly acidic mobile phase that has been described[4] in the analysis of fenbendazole residues in milk resulted in chromatograms with broad and tailing peaks. Attempts to resolve this problem by applying a flow rate program similar to that suggested[2,3] for the analysis of fenbendazole and its metabolites in plasma, urine, feces, and tissue homogenates, helped somewhat but failed to provide an acceptable procedure. In those ionization enhancement systems, where silanophilic interactions of the protonated analytes with the stationary phase occurs, peak distortion was more pronounced for the late eluted fenbendazole (Fig. 2A). To eliminate peak tailing and improve the chromatographic efficiency, a pH 7 mobile phase with similar elution strength was used instead.

However, excessive retention of all analytes was noted due to suppression of their ionization at this pH value. By increasing the elution strength of the mobile phase, retention could be drastically shortened but the tailing of the peaks could not be totally eliminated [Fig. 2B]. It might be of interest to note, that in these ion-suppression conditions the elution order of the p-hydroxy and sulphone metabolites reversed, the latter eluting now before the former.

Figure 2 also shows that, using a pH 7 mobile phase, peaks of both fenbendazole and p-hydroxyfenbendazole are higher than those expected on the basis of the recorded response with the pH 2.2 mobile phase. This was atributed to the significant absorbance difference between the protonated and unprotonated forms of these compounds. Upon acidification, the absorbance of both fenbendazole and p-hydroxyfenbendazole, unlike the other metabolites, undergoes a remarkable reduction (78-80 %).

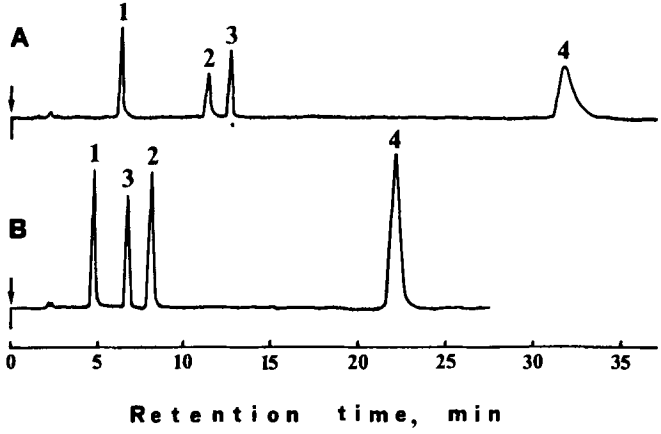

Figure 2. Chromatograms of a mixed standard working solution (2.5 μg/mL fenbendazole sulphoxide, 3.0 μg/mL p-hydroxyfenbendazole, 2.4 μg/mL fenbendazole sulphone, and 10.0 μg/mL fenbendazole) run with a mobile phase consisting of (A) acetonitrile-0.01 M phosphoric acid (30:70, v/v) or (A) acetonitrile-0.01 M phosphate buffer, pH 7, (40:60, v/v). Other LC conditions: column 250x4.6 mm, Nucleosil 120, C_{18}, 5μm; temperature, 30°C; flow rate, 1 mL/min; wavelength 290 nm; detection sensitivity, 0.05 a.u.f.s.; chart speed, 5 mm/min; injection volume, 10 μl. Peak identification: fenbendazole sulphoxide (1), p-hydroxyfenbendazole (2), fenbendazole sulphone (3), and fenbendazole (4).

To inactivate the residual free silanol action on the silica-based C_{18} packing material and eliminate peak tailing, modification of the LC partitioning process through addition to the mobile phase of various pairing agents was examined. In this investigation, an acidic mobile phase that consisted of acetonitrile and 0.01 M phosphoric acid (30/70, v/v) was selected as reference point. The effect of the pairing agents examined (type and concentration) on the retention and peak shapes of all analytes is best illustrated in Figure 3. In absence of ion-pair reagents, low height and badly tailed peaks appeared due, obviously, to strong silanophilic interactions of the protonated analytes with the stationary phase (Fig. 3A). Addition of negatively charged octanesulphonate ions in the mobile phase had a remarkable effect on both the shape and the elution order of the peaks (Fig. 3B). Despite the high increase of retention noted due to ion-pairing of the octanesulphonate anions with the positively charged benzimidazoles to more hydrophobic forms, peak heights were not significantly decreased, and peak distortion was reduced.

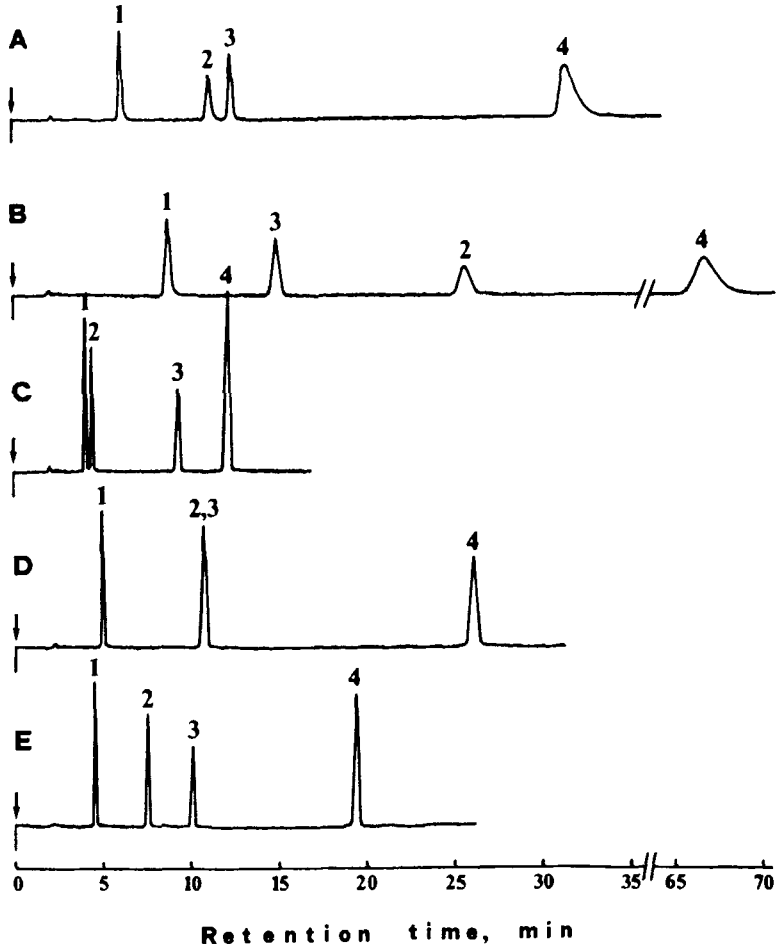

Figure 3. Chromatograms of a mixed standard working solution (concentrations as in Figure 2) run with an acetonitrile-0.01 M phosphoric acid (30:70, v/v) mobile phase containing or not ion-pair reagents; (A) without reagents; (B) with 5 mM octanesulphonate reagent; (C) with 5 mM tetrabutylammonium reagent; (D) with 5 mM octanesulphonate and 5 mM tetrabutylammonium reagents; (E) with 2.5 mM octanesulphonate and 5 mM tetrabutylammonium reagents.). Other LC conditions as in Figure 2.

Nevertheless, the tailing of the peaks could not be totally eliminated. Figure 3B also shows that octanesulphonate is an efficient means for regulating the elution order of the p-hydroxy and sulphone metabolites, as the former can elute far beyond the latter upon addition of the ion-pair reagent.

Figure 3C demonstrates that the most significant change in the chromatographic profile of the analytes is produced when positively charged tetrabutylammonium ions are added in the mobile phase. Retention was greatly decreased, peak tailing was totally eliminated, and peak heights were spectacularly increased. These effects may partly, at least, be attributed to efficient masking of the surface free silanol groups by the tetrabutylammonium reagent.[12,13] Electrostatic repulsion of the protonated analytes by the tetrabutylammonium cations adsorbed on to the octadecylsilica surface could also contribute to these effects.[14,15] At these conditions, however, p-hydroxyfenbendazole eluted quite close to the sulphoxide metabolite, as the elution time of the former had been largely shortened.

Considering that both octanesulphonate and tetrabutylammonium pairing ions could variably influence the chromatographic behavior of the fenbendazole metabolites, co-addition of these reagents in the mobile phase was investigated in an effort to achieve a desirable degree of separation between all analytes in a minimum time. It was found that addition of equimolar concentrations of these reagents could separate the sulphoxide and the p-hydroxy metabolites but the resolution of the latter from the sulphone metabolite was destroyed (Fig. 3D). In contrast, excellent resolution could be made possible when the molar concentration of the added octanesulphonate was half the concentration of the tetrabutylammonium reagent (Fig. 3E).

The retention behavior observed in this study, in which case the mobile phase in addition to tetrabutylammonium cations contains equal or lower concentration of octanesulphonate anions, is difficult to explain. The chromatographic mechanism in such systems has not yet been elucidated. The octanesulphonates might interact with both the anti-tailing tetrabutylammonium and solute ions; further, the two opposite charged surfactants might be co-adsorbed on to the column material.[16,17] Figure 3 indicates that the affinity of tetrabutylammonium cations to residual silanols should be more pronounced than to octanesulphonate anions, as the anti-tailing effect of the tetrabutylammonium reagent is not reduced by the presence of octanesulphonates. Figure 3 also suggests that negatively charged pairing ions capable to form ion pairs with solute cations are present, even if their concentration is equal to that of the positively charged pairing ions. Thus, it might be expected that the effect of variation in the concentration of the octanesulphonates would be as in reversed phase ion pair chromatography; further enhancement of retention occurs when the concentration of octanesulphonate anions increases. These observations that lend support to previous findings[11,18] may help in better understanding the retention mechanisms involved in ion-pair chromatography.

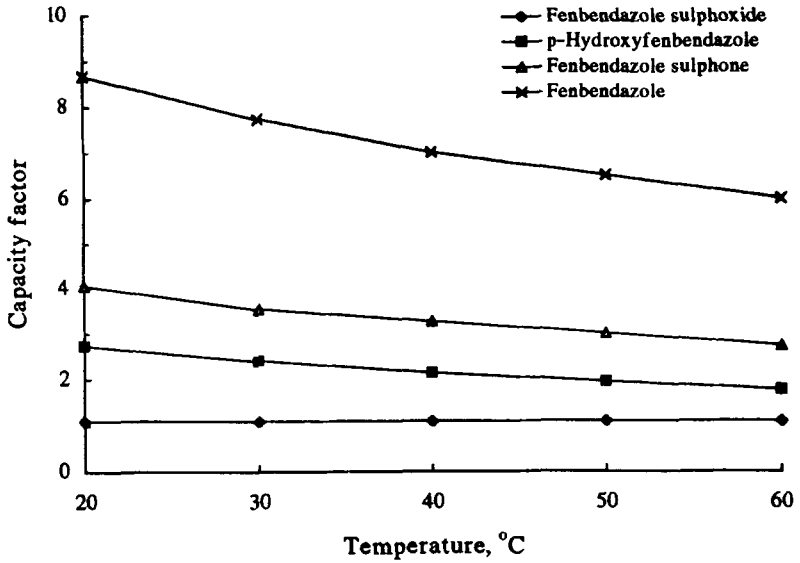

Figure 4. Effect of column temperature on the capacity factors of fenbendazole and its metabolites.

The effect of column temperature on retention time, when the mobile phase contains the optimized ratio of 2.5 mM octanesulphonate and 5 mM tetrabutylammonium reagents, was also investigated. Figure 4 shows that progressive reduction in retention for fenbendazole and its p-hydroxy and sulphone metabolites occurs when column temperature is increased up to 60°C, which is typical behavior for a reversed phase system. The retention, however, of the early eluted sulphoxide metabolite could not be affected by temperature change, a finding suggesting that control of temperature may aid in specific ion-pair separations.

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Received June 10, 1996
Accepted July 4, 1996
Manuscript 4213