DISCUSSION

The artificial sweetener consists of saccharin sodium and sodium bicarbonate. Acetic acid in the eluent neutralizes both. Acetic acid in the eluent increases the retention (capacity factor) of saccharin by suppressing ionization and also results in a much sharper peak. If saccharin is allowed to elute in its ionic form, very little retention occurs and little use is made of the separating capabilities of the column.

Before an assay can be considered qualified for a stability-indicating program, its specificity characteristics must be proven. Specificity in this method is provided mainly by the liquid chromatographic column. It is clearly not proper to assume specificity simply because a chromatographic process is involved. For this study, the proposed method was challenged in three ways.

First, a literature search was conducted to determine the documented impurities and degradation products of the active drug saccharin. These compounds were subjected to the column under the assay conditions and were all innocuous to the assay. These compounds must be added directly to the sample solution so that possible matrix effects are eliminated.

Second, a forced degraded sample of the actual product was assayed for two reasons: (*a*) the actual decomposition products in the drug matrix may be different from those postulated in the literature and (*b*) interferences by degradation products of the excipients are possible. When the assay passes these conditions, it is more likely to prevail during the shelflife of the product.

Third, the forced degraded sample assay, whenever possible, should be compared with a completely independent assay based on different analytical principles. If possible, this reference method should be of demonstrated accuracy. When the results of the two methods agree within the expressed objectives, the method developer can be confident of the reliability of the new method. These three approaches are by no means completely rigorous but can be regarded as minimum criteria for a proposed stability-indicating method.

The general procedure given for other pharmaceutical preparations was applied to mouthwashes, analgesic liquids, and antidiarrhetics. Occasionally, such samples can be injected after dilution, addition of the internal standard, and clarification. Generally, however, ether extraction is required because of the low saccharin levels in the formulations. The ether extraction technique also is preferable because of the added selectivity of the extraction step and the extended column life resulting from injection of a cleaned-up sample solution.

Because of pharmaceutical formulation diversity, components other than saccharin are frequently extracted and the resulting chromatograms are less simple than those of the artificial sweetener. However, interferences often may be eliminated by proper pH and methanol adjustments in the eluent. The selectivity of the chromatographic process provides enough resolution so that the saccharin assay can be carried out in the presence of other ingredients such as salicylic acid and alkyl parabens. Injections can be made every 5 min, and about 30 samples of artificial sweetener can be analyzed per 8-hr day. Fewer samples of the other pharmaceutical formulations can be analyzed because of the lengthier sample-handling procedure, but this procedure is necessary for almost any technique.

In summary, this reversed-phase liquid chromatographic system provides a precise, accurate, and specific saccharin assay with high sample throughput. The procedure is applicable to a wide range of pharmaceuticals, especially for a stability-indicating program.

REFERENCES

(1) *Anal. Abstr.*, **26**, 3261 (June 1974).

(2) I. K. Shih and F. W. Teare, *Can. J. Pharm. Sci.*, **1**, 35 (1966).

(3) J. R. Markus, *J. Assoc. Off. Anal. Chem.*, **56**, 162 (1973).

(4) S. P. Mukherji, *Analyst*, **85**, 25 (1960).

(5) A. M. Lasheen, *Proc. Am. Soc. Hortic. Sci.*, **77**, 135 (1961).

(6) T. Korbelak, *J. Assoc. Off. Anal. Chem.*, **52**, 487 (1969).

(7) N. Hazemoto, N. Kamo, and Y. Kobatake, *ibid.*, **57**, 1205 (1974).

(8) H. Jacobson, *Ann. N.Y. Acad. Sci.*, **153**, 486 (1968).

(9) *Anal. Abstr.*, **30**, 6E43 (June 1976).

(10) *Ibid.*, **14**, 7864 (Dec. 1967).

(11) E. M. Ratchip and V. Viswanathan, *J. Pharm. Sci.*, **64**, 133 (1975).

(12) H. B. S. Conacher and R. C. O'Brien, *J. Assoc. Off. Anal. Chem.*, **54**, 1135 (1971).

(13) R. J. Daun, *ibid.*, **54**, 1140 (1971).

(14) R. Gerstl and K. Ranfft, *Z. Anal. Chem.*, **258**, 110 (1972).

(15) M. W. Couch, N. P. Das, K. N. Scott, C. M. Williams, and R. L. Foltz, *Biochem. Med.*, **8**, 362 (1973).

(16) J. J. Nelson, *J. Chromatogr. Sci.*, **11**, 28 (1973).

(17) D. S. Smyly, B. S. Woodward, and E. C. Conrad, *J. Assoc. Off. Anal. Chem.*, **59**, 14 (1976).

(18) B. Stavric, R. Lacombe, J. R. Watson, and I. C. Munro, *ibid.*, **57**, 678 (1974).

(19) R. E. King and J. S. Wragg, *J. Pharm. Pharmacol. Suppl.*, **18**, 22 (1966).

(20) M. N. Kazennova, *Optikomekh. Prom.* (12), 42 (1972); through *Anal. Abstr.*, **26**, 3261 (June 1974).

High-Performance Liquid Chromatographic Assay for the Anthelmintic Agent Mebendazole in Human Plasma

K. B. ALTON ˣ, J. E. PATRICK, and J. L. McGUIRE

Received June 30, 1976, from the *Drug Disposition Section, Ortho Pharmaceutical Corporation, Raritan, NJ 08869.* Accepted for publication January 16, 1979.

Abstract □ A rapid and specific high-performance liquid chromatographic (HPLC) assay for quantitative plasma mebendazole determination is described. After a simple extraction, the compound was analyzed by HPLC using a reversed-phase column and a UV detector (313 nm). Quantitation was accomplished using an internal standard; peak area ratios were determined with an integrating computer. The average mebendazole recovery over a concentration range of 0.01–0.20 μg/ml was 75.9 ± 3.8% *SD*, and the maximum assay sensitivity was ~10 ng/ml.

Keyphrases □ High-performance liquid chromatography—analysis, mebendazole in human plasma □ Mebendazole—analysis, high-performance liquid chromatography, in human plasma □ Anthelmintic agents—mebendazole, high-performance liquid chromatographic analysis in human plasma

Mebendazole[1] (I), methyl 5-benzoyl-2-benzimidazolecarbamate, is a broad spectrum anthelmintic agent (1–3). Initial absorption studies in humans (1) suggested that 6–10% of the radioactivity was recovered in the urine following 5–7 mg po of ^{14}C-mebendazole (18.5–22.7 μCi). Approximately 2.5% of this urinary radioactivity was unconjugated mebendazole, about 20% was the decarbamated metabolite, and 75% was of undetermined identity. Plasma radioactivity levels remained low throughout the study. There are no published methods sensitive enough to

[1] Vermox tablets, Ortho Pharmaceutical Corp.

0022-3549/79/0700-0880$01.00/0

measure plasma levels of the parent compound following oral administration of mebendazole tablets.

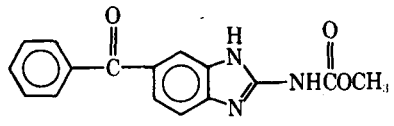
This report describes a simple and specific high-performance liquid chromatographic (HPLC) method for the rapid determination of plasma mebendazole. This assay may have the sensitivity (~0.01 μg/ml) and precision needed to measure unchanged drug levels after chronic high-dose administration of mebendazole to patients being treated for *Echinococcus multilocularis* cysts.

EXPERIMENTAL

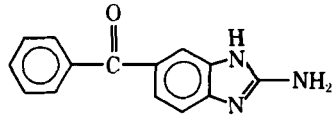
Apparatus—Analyses were performed on a liquid chromatograph[2] operated at ambient temperature and equipped with a differential UV (313 nm) detector[3]. Separations were performed on a 300 × 3.0-mm (i.d.) reversed-phase column[4]. Samples were introduced onto the column through a septumless injector[5] with a 25-μl syringe[6]. Chromatograms were traced on a strip-chart recorder[7], and peak area integration was performed by an integrating computer[8] interfaced with the detector by an analog–digital converter[9]. Analog signal modification to reduce noise from the UV detector was achieved with an electronic filter[10].

Reagents and Solvents—Mebendazole (I), three known human urinary metabolites of mebendazole [2-amino-5-benzoylbenzimidazole (II), 5-benzoyl-2-hydroxybenzimidazole (III), and methyl 5-(α-hydroxybenzyl)-2-benzimidazolecarbamate (IV)], and the internal standard methyl 5-(4-fluorobenzoyl)-2-benzimidazolecarbamate (V, flubendazole) were used as received[11]. All chemicals and reagents except acetonitrile[12] were reagent grade.

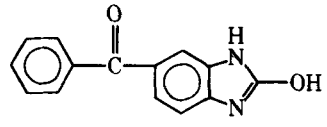
Chromatographic Conditions—The mobile phase was 0.05 *M* KH_2PO_4–NaOH buffer (pH 6.0)–acetonitrile (73:27 v/v), and the flow rate was adjusted to 2.5 ml/min (~2500 psi). The solvent mixture was prepared daily and degassed under reduced pressure before use. A stainless steel precolumn[13] (3 × 0.32 cm o.d.) was used to minimize the detrimental effect of any irreversibly adsorbed plasma-extracted material on the performance of the microparticulate reversed-phase column.



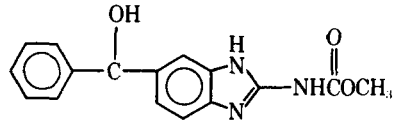
I



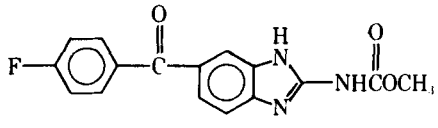
II



III



IV



V

Table I—Recovery of Mebendazole from Human Plasma at Various Concentrations

Theoretical Plasma Concentration, μg/ml	Average Observed Plasma Concentration, μg/ml	*n*	Average Recovery[a], %
0.010	0.0757	7	75.7 ± 9.3
0.025	0.0187	6	74.9 ± 5.1
0.050	0.0389	9	77.9 ± 1.9
0.100	0.0754	13	75.4 ± 1.4
0.200	0.151	6	75.4 ± 1.5
		$\bar{x}$	75.9 ± 3.8[b]

[a] $\bar{x} \pm SD$. [b] $\overline{CV}$ = 5.09%.

Extraction Procedure—Aliquots (2.0 ml) of plasma were transferred to 15-ml test tubes (16 × 125 mm) fitted with polytef-lined screw caps, diluted with 2.0 ml of 0.05 *M* KH_2PO_4–NaOH buffer (pH 7.0), and extracted with 7.0 ml of ethyl acetate. The mixture was shaken vigorously, and the phases were separated by centrifugation (2 min at ~1500×*g*). The organic layer was removed with a Pasteur pipet and transferred to another 15-ml screw-capped tube. A second extraction was performed similarly. The ethyl acetate layers were pooled for each sample and taken to dryness under a nitrogen stream in a water bath (55°). An aliquot (2.0 ml) of 0.001 *N* HCl was added to the ethyl acetate residue and mixed with a vortex mixer. The hydrochloric acid solution was then extracted twice with 6.0 ml of petroleum ether (bp 30–60°) and centrifuged to obtain a clear supernate, and the upper layers were discarded after aspiration. The remaining aqueous fraction was alkalinized with 2.0 ml of 0.01 *N* NaOH, and this mixture was extracted twice with ethyl acetate.

The final residues were transferred with ethyl acetate to 2.5-ml glass conical centrifuge tubes and concentrated with two ethyl acetate washings under a nitrogen stream in a water bath (55°). The resulting residue was dissolved in 60 μl of ethyl acetate, and aliquots (20 μl) were injected onto the chromatograph for analyses.

Extraction Efficiency—Venous blood from several untreated volunteers was drawn into heparinized containers[14] and centrifuged to generate a plasma pool. The efficiency of extracting the drug from plasma was established using the following procedure. Known amounts of mebendazole and of the internal standard, flubendazole, both of which were dissolved in methanol–formic acid (described later), were added to drug-free plasma samples (15–25 ml each) to achieve mebendazole concentrations of 0.01, 0.025, 0.05, 0.10, and 0.20 μg/ml and a constant flubendazole concentration of 0.15 μg/ml for each sample. Aliquots (2.0 ml) of each plasma sample were transferred to 15-ml test tubes and stored frozen (−20°) until extracted as described.

All samples ($n \geq 6$) at each concentration were analyzed in duplicate. Mebendazole quantitation was accomplished by use of the described internal standard method.

Calibration and Standard Preparation—Mebendazole (25.0 mg) was dissolved in ~10 ml of formic acid (98%) and diluted to volume with methanol in a 25.0-ml volumetric flask to yield a concentration of 1.0 μg/μl. Three additional standard solutions containing 0.10, 0.01, and 0.001 μg/μl of mebendazole were prepared by serial dilution of the 1.0-μg/μl stock solution with methanol. Standard solutions of flubendazole (1.0, 0.1, and 0.01 μg/μl) were prepared in the same manner.

The internal standard method contained in the software section of the computer is predicated on a linear relationship between micrograms of mebendazole injected and the peak area ratio of mebendazole to the internal standard. Calibration solutions were prepared in the following manner. Aliquots (100, 71, 50, 33, and 25 μl) of the 0.1-μg/μl internal standard solution were transferred to glass vials and taken to dryness. These samples were diluted with 1.0 ml of the mebendazole standard solution (0.001 μg/μl) so that a constant amount of flubendazole (0.05 μg) and 0.005, 0.007, 0.01, 0.015, and 0.02 μg of mebendazole were delivered in 5, 7, 10, 15, and 20 μl of solution. The linearity of the calibration curve was initially evaluated by repeated ($n \geq 5$) injections of each solution. Thereafter, an average calibration response factor was established by the computer following triplicate injections (15 μl) of a calibration

[2] Model ALC 202/204, Waters Associates, Milford, Mass.
[3] Model 440, Waters Associates, Milford, Mass.
[4] μBondapak C-18, Waters Associates, Milford, Mass.
[5] Model U6K, Waters Associates, Milford, Mass.
[6] Pressure-Lok B-110, Precision Sampling Corp., Baton Rouge, La.
[7] Model 300, Linear Instruments, Irvine, Calif.
[8] Model 3352B, Hewlett-Packard, Avondale, Pa.
[9] Model 18652A, Hewlett-Packard, Avondale, Pa.
[10] Model 1021 A, Spectrum Scientific Corp., Newark, Del.
[11] Mebendazole, flubendazole, and metabolite standards were synthesized and supplied by Janssen Pharmaceutica, Beerse, Belgium.
[12] Glass distilled, Burdick & Jackson Laboratories, Muskegon, Mich.
[13] Packed with C-18 Corasil II, Waters Associates, Milford, Mass.
[14] Vacutainers, Becton Dickenson, Rutherford, N.J.

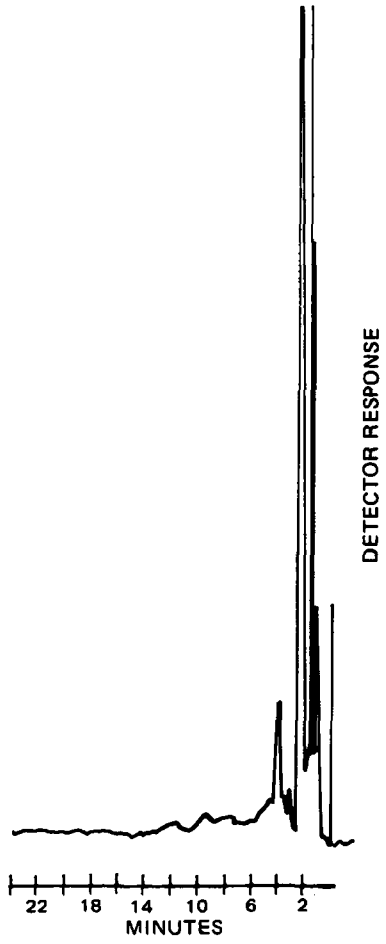


Figure 1—*Liquid chromatogram of an extract from drug-free plasma. The mobile phase consisted of 0.05 M KH_2PO_4–NaOH buffer (pH 6.0)–acetonitrile (73:27 v/v) with the flow rate adjusted to 2.5 ml/min.*

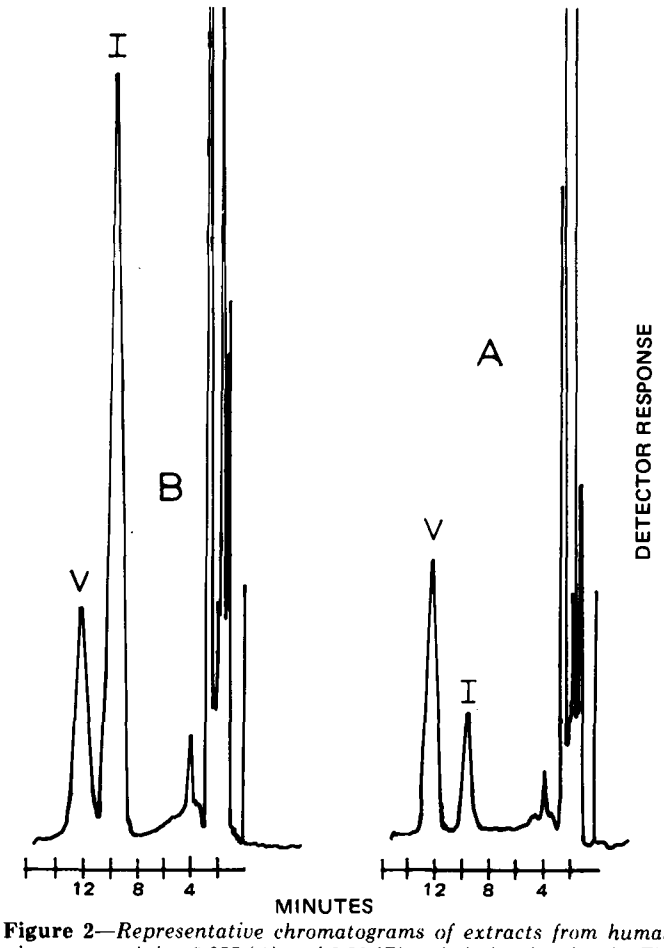


Figure 2—*Representative chromatograms of extracts from human plasma containing 0.025 (A) and 0.20 (B) μg/ml of mebendazole. The amount of the internal standard added to the plasma was 0.15 μg/ml. The detector response was at 0.005 aufs.*

solution containing 0.015 μg of mebendazole and 0.05 μg of the internal standard.

Specificity—Assay specificity was determined by injecting solutions of mebendazole (I) and its potential metabolites, II, III, and IV (~0.1 μg/μl in methanol), onto the HPLC column. A portion of an ethyl acetate extract (20 μl/60 μl) from a drug-free plasma sample also was chromatographically evaluated for the presence of any ethyl acetate-extractable, UV-absorbing (313 nm) material that might interfere with the measurement of mebendazole or flubendazole.

RESULTS AND DISCUSSION

The average drug recovery from plasma samples to which mebendazole had been added was 75.9 ± 3.8% *SD* (Table I) with an average precision (*CV*) of 5.09%. Linear regression analysis of percent recovery *versus* plasma concentration revealed no statistically significant ($p = 0.7143$) slope (β). One-way analysis of variance of the recovery data from each mebendazole concentration demonstrated no significant differences among the groups. These data suggest that extraction efficiencies are not concentration dependent over the investigated plasma drug range. The average internal standard recovery was 94.1 ± 2.88% *SD* with a coefficient of variation equal to 3.06%.

Even though the λ_{max} observed for mebendazole in methanol was 246 nm, detection at 313 nm was particularly advantageous since the ethyl acetate extracts from drug-free plasma were free of interfering UV-absorbing peaks (313 nm) (Fig. 1). Extraction of the acidified residue with petroleum ether aided in eliminating lipophilic material, which might otherwise become highly bound to the column and interfere with analyses.

Baseline resolution between mebendazole (I) and the internal standard, flubendazole (V), was achieved under the chromatographic conditions described. Compounds II, III, and IV, although not well separated from each other, were separated completely from I and V. The observed retention times (t_r) for I, II, III, IV, and V were 10.03, 5.61, 4.89, 4.89, and 12.64 min, respectively. Representative chromatograms of extracts from plasma containing 0.025 and 0.20 μg/ml of mebendazole are shown in Fig. 2.

Linear regression analysis of the curve described by plotting the area ratio (*y*-axis) of mebendazole over flubendazole *versus* micrograms of mebendazole injected (*x*-axis) demonstrated linearity ($r^2 = 0.9803$) over the concentration range evaluated (0.005–0.02 μg/injection). The slope of the line was calculated to be 21.908 μg^{-1}, and the *y*-intercept was calculated to be 0.021. The calculated *x*-intercept was equivalent to −0.96 ng of mebendazole/injection. This value was used as a background correction factor in mebendazole recovery calculations. Over a 2-month period of analyses, the slope of this line demonstrated little change ($CV = 4.8\%$, $n = 56$).

In summary, an assay was developed that can be used to measure plasma mebendazole levels as low as 0.01 μg/ml. The method is both rapid and specific, and its sensitivity indicates that it might be suitable for measuring unchanged drug levels after chronic administration of mebendazole tablets to patients being treated for *E. multilocularis* cysts.

REFERENCES

(1) J. P. Brugmans, D. Thienpont, I. Van Wijngaarden, O. F. Vanparijs, V. L. Schuermans, and H. L. Lauwers, *J. Am. Med. Assoc.*, **217**, 313 (1971).

(2) E. Fierlafijn, *Bruxelles-Med.*, **51**, 605 (1971).

(3) J. F. F. Callear and R. M. S. Neave, *Br. Vet. J.*, **127**, XLI (1971).

ACKNOWLEDGMENTS

The authors thank Dr. J. Heykants of Janssen Pharmaceutica, Beerse, Belgium, for providing samples of mebendazole, flubendazole, and metabolite standards. They also thank Mr. J. A. Shigo for technical assistance and Mr. R. Chen for statistical services.