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TWO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATIONS FOR MEBENDAZOLE AND ITS METABOLITES IN HUMAN PLASMA USING A RAPID SEP PAK C18 EXTRACTION

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SUMMARY

A rapid extraction procedure for mebendazole and its metabolites from plasma using Sep Pak C18 is described. This method eliminates the need for solvent extractions as such.

Two reversed-phase high-performance liquid chromatographic determinations for these extracts, one isocratic elution and the other gradient elution, using an analytical wavelength of 254 nm are also presented.

The gradient elution system provides superior resolution of these compounds and consequently has improved determination limits. For mebendazole the determination limits are 20 ng/ml (isocratic system) and 10 ng/ml (gradient system).

INTRODUCTION

Mebendazole is a broad spectrum anthelmintic drug which is tolerated well in animals [1]. There is growing evidence that mebendazole could be a useful chemotherapeutic agent in the management of hydatid disease in man, especially in cases where the cyst is surgically inaccessible or where spillage has occurred during surgical removal [2—8].

The very low water solubility of mebendazole (ca. 0.7 mg/l) prevents its intravenous administration and renders it poorly absorbed when administered orally. As a consequence the blood levels of mebendazole achieved after oral dosing are often difficult to quantify. High-performance liquid chromatography (HPLC) can detect and quantify mebendazole and its metabolites in human biological fluids in sub-microgram per ml concentrations. It is the only practical analytical system available for this determination.

Metabolism and pharmacokinetics studies with mebendazole during high level chronic dosing (ca. 40 mg/kg/day), as would be necessary in the treatment of hydatid disease, have not been reported in the literature, however in vitro metabolism studies have been performed in pig, rat and dog liver fractions [9].

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The major metabolite of mebendazole (methyl 5-benzoylbenzimidazole carbamate, I, Fig. 1) was found to be methyl 5-(α-hydroxybenzyl)-2-benzimidazole carbamate (III). A second metabolite was identified as 2-amino-5-benzoylbenzimidazole (II) and a minor metabolite was tentatively identified as 2-amino-5-(α-hydroxybenzyl)-benzimidazole (IV). An in vivo study [10] found compound II to be the major metabolite of mebendazole in man.

Two HPLC analyses for mebendazole in human plasma have been reported recently. One method [11] involved multiple extractions, washes and pH adjustments and used an analytical wavelength of 313 nm. The other method [12] was a simple solvent extraction which used an analytical wavelength of 307 nm. Compounds III and IV have very low molar extinction coefficients in the region of 310 nm and therefore these wavelengths are not suitable for their detection.

This work describes a rapid extraction procedure which eliminates solvent extractions as such but still produces a clean extract from human plasma suitable for HPLC analysis. Two HPLC systems are described which separate and quantitate mebendazole and its metabolites using an analytical wavelength of 254 nm and ethyl 5-benzoylbenzimidazole carbamate (V) as an internal standard.

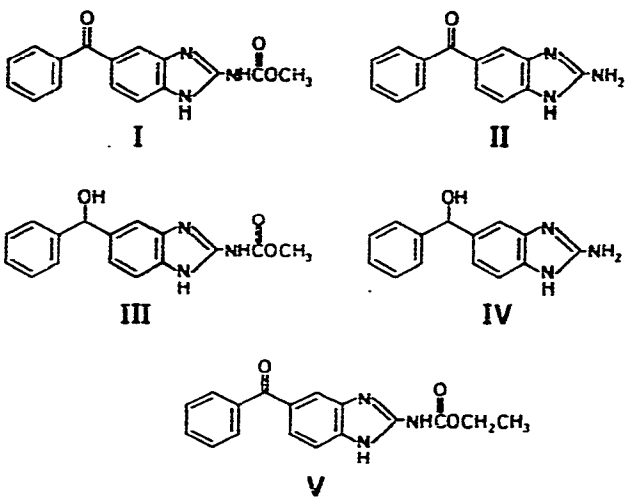

Fig. 1. Mebendazole (I), its metabolites (II, III and IV) and the internal standard (V).

EXPERIMENTAL

Materials

Mebendazole (I) was supplied by Ethnor (Sydney, Australia). Metabolite II was made by alkaline hydrolysis of I. Metabolites III and IV were made by sodium borohydride reduction of I and II respectively. The internal standard (V) was supplied by Mr E. Lacey of this department. Purity was confirmed by thin-layer chromatography (TLC), HPLC and mass spectrum analyses. Methanol and dimethyl sulphoxide (DMSO) (Ajax Chemicals, Sydney, Australia) were of analytical reagent grade. Phosphoric acid, hydrochloric acid,

ammonia solution and sodium carbonate were all of laboratory reagent grade and were used without further purification. Plasma was obtained from drug-free healthy male volunteers.

Apparatus

The instruments used were an Altex Model 322 MP high-performance liquid chromatograph (Altex Scientific, Berkeley, CA, U.S.A.) equipped with a fixed-wavelength detector (254 nm, 8-μl flow cell), a Rheodyne (Berkeley, CA, U.S.A.) Model 7120 injection valve fitted with a 20-μl sampling loop, and a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3380A integrator recorder.

Extractions were performed using Sep Pak C18 cartridges (Waters Assoc., Milford, MA, U.S.A.).

Column

The chromatographic column (250 × 4.6 mm I.D.) contained LiChrosorb RP-8 10-μm reversed-phase packing (E. Merck, Darmstadt, G.F.R.) and was supplied packed by Brownlee Labs. (Santa Clara, CA, U.S.A.). A precolumn (35 × 3.2 mm I.D.), dry-packed with Corasil C18 (Waters Assoc.) was fitted to the system.

Mobile phase

The mobile phase consisted of methanol—distilled water (55 : 45, pump A) and methanol—aqueous ammonium phosphate 0.05 *M*, pH 5.5 (55 : 45, pump B).

Operating conditions

The flow-rate was 1.7 ml/min which produced a column pressure of 1200—1300 p.s.i. (ca. 8.2—9.0 MPa). Isocratic elution was performed with pump A providing 67% of the mobile phase and pump B the balance. Gradient elution commenced with pump A supplying 100% of the mobile phase. At 7 min a curved gradient began which brought pump B up to 45% at 13.2 min and then held these conditions up to 20 min (Fig. 2).

The detector sensitivity was set at 0.005 a.u.f.s. The chromatograms were attenuated at the integrator when necessary.

All operations were carried out at room temperature (ca. 20°C).

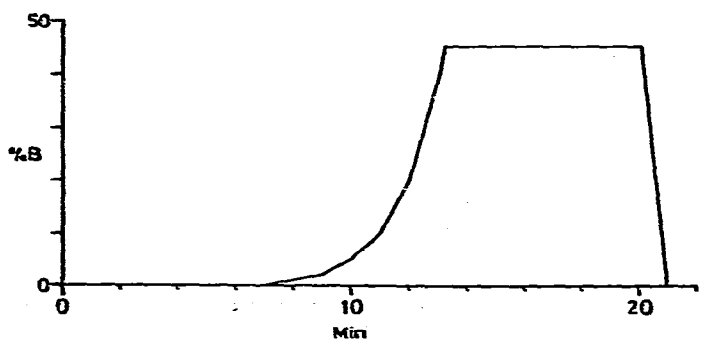

Fig. 2. The gradient elution profile. The contribution of pump B (%B) to the flow-rate (1.7 ml/min) versus time is plotted.

TABLE I

DEVELOPMENT OF EXTRACTION METHOD

Methanol fraction		Recovered (%)				
No.	Volume (ml)	I	II	III	IV	V
1,2,3,4	0.1	—	—	—	—	—
5	0.1	0.02	—	0.3	—	—
6	0.5	71.2	70.1	71.3	65.6	63.0
7	0.5	16.3	12.7	11.3	20.6	16.7
8	0.5	—	—	—	3.4	—
9	2.0	—	—	—	—	—
Σ5,6,7,8	1.6	87.52	82.8	82.9	89.6	79.7

TABLE II

RECOVERY STUDY

N (number of assays) = 3 in each case.

Compound	Theoretical content (μg)	Recovery mean ± S.D. (μg)	Recovery (%)
I	5.20	4.55 ± 0.128	87.5
	1.97	1.75 ± 0.064	88.8
	0.49	0.41 ± 0.025	83.7
	0.20	0.18 ± 0.013	90.0
II	4.90	4.05 ± 0.123	82.7
	1.97	1.45 ± 0.021	73.6
	0.49	0.31 ± 0.005	63.3
	0.20	0.15 ± 0.020	75.0
III	4.95	4.10 ± 0.006	82.8
	1.83	1.58 ± 0.063	86.3
	0.46	0.36 ± 0.013	78.3
	0.18	0.14 ± 0.034	77.8*
IV	5.18	4.64 ± 0.279	89.6
	2.06	1.75 ± 0.080	85.0
	0.52	0.30 ± 0.077	57.7*
	0.21	0.32 ± 0.059	152.4*
V	4.50	3.61 ± 0.049	80.2
	1.67	1.32 ± 0.076	79.0
	0.42	0.27 ± 0.025	64.3
	0.17	0.11 ± 0.009	64.7

*These values, when converted to μg/ml, are below the determination limits defined in Table III.

Extraction procedure and standard curve preparation

Standard solutions and serial dilutions of compounds I, II, III, IV and V were made in DMSO. A standard solution containing all five compounds was used to calibrate the integrator.

Plasma samples (5 ml) were spiked with varying amounts of the five compounds for the recovery studies (Table I, Table II). The standard curves were prepared by spiking 5-ml plasma samples with varying amounts of compounds I, II, III and IV, and with 5.0 μg of compound V as internal standard, in the range 30 μg to 10 ng (equivalent to concentrations of 6 μg/ml to 2 ng/ml). Samples were adjusted to pH 6 with dilute hydrochloric acid or sodium carbonate solution and extracted by passing through a Sep Pak C18 cartridge fitted to a Luer Lok glass syringe.

The Sep Pak C18 cartridge was prepared by flushing with 5 ml of methanol followed by 5 ml of aqueous ammonium phosphate 0.017 *M*, pH 5.5. After the spiked plasma was passed through the cartridge it was washed with 20 ml of distilled water, 0.5 ml of 40% methanol in distilled water and 0.4 ml of methanol. The next 1.6 ml of methanol eluted the five compounds from the cartridge. After washing with a further 10 ml of methanol the cartridge was ready for re-use.

The 1.6-ml methanol fractions were evaporated to dryness in pointed glass tubes on a water bath and then the residues were redissolved in 100 μl of DMSO. Aliquots of 20 μl were injected into the HPLC system.

Calculation of results

The extraction development and recovery results were calculated using the HP3380A external standard routine which computed the amount of each compound injected from the calibration data. The amounts present in 100 μl were then expressed as percentages of the amounts initially added. Results for the standard curves were calculated using the HP3380A internal standard routine which computed peak area ratios with respect to the internal standard and then multiplied the result by the amount of internal standard added to give the amount of each compound in the entire sample. A control plasma extract containing only the internal standard was used to estimate the background levels associated with each compound. These values were used as corrections for the data obtained before any statistical analyses were carried out.

RESULTS AND DISCUSSION

The Sep Pak C18 extraction development is detailed in Table I. In subsequent extractions the fractions containing compounds I, II, III, IV and V were collected as a single fraction. Details of the extraction efficiency of this method are recorded in Table II. The use of the Sep Pak C18 cartridge for plasma extractions had several advantages over the solvent extraction methods. It provided chromatographically cleaner extracts because of the partial separation which it produced, it removed any materials which otherwise might have adsorbed irreversibly to the chromatographic column, and it effected a significant time saving per extraction because it was a single operation. The extraction efficiency of this method was at least equal to that of the solvent extraction methods.

Contrary to the manufacturer's recommendation we found that a Sep Pak C18 cartridge could be re-used a number of times without any loss of performance.

Linear regression analyses were performed on the data from the two chromatographic systems. The regression lines were constructed from a minimum of 28 data points. Most points were duplicates but larger numbers of replicates were prepared in the region of the minimum determinable concentrations (S.D. $\leq$ 10%). Table III presents the correlation coefficients for mebendazole and its metabolites in the two systems and their minimum determinable concentrations. Typical chromatograms are shown in Fig. 3 and the gradient profile used is presented in Fig. 2.

The retention times and resolution of compounds II and IV in these systems were very dependent on the mobile phase electrolyte concentration, as indicated in Table IV. Variations to the percentages of mobile phases A and B in the isocratic system could be made, if necessary, to optimize the resolution of compounds II, III and IV.

The gradient elution separation was based on the observation that in the absence of electrolytes, compounds II and IV travelled through this system very slowly while compounds I, III and V were unaffected. By maintaining a constant methanol concentration in the mobile phase throughout the gradient, the problems associated with baseline shifts were avoided. Because the resolution in the gradient elution system was particularly electrolyte dependent, it was necessary to adequately re-equilibrate the column before each run. Re-equilibration was achieved by pumping 25 column (plus pre-column) volumes of electrolyte-free mobile phase through the system. The flow-rate was increased during this operation to shorten the re-equilibration time.

TABLE III

CORRELATION COEFFICIENTS AND DETERMINATION LIMITS

S.D. $\leq$ 10%.

Compound	Correlation coefficient	N^*	Determination limit (μg/ml)	N^{**}
Isocratic elution				
I	0.9982	42	0.02	4
II	0.9926	39	0.06	3
III	0.9975	28	0.06	3
IV	0.9787	30	0.56	4
Gradient elution				
I	0.9979	39	0.01	3
II	0.9914	41	0.03	4
III	0.9986	30	0.06	4
IV	0.9959	30	0.10	4

*Number of points in regression analysis.

**Number of points at determination limit.

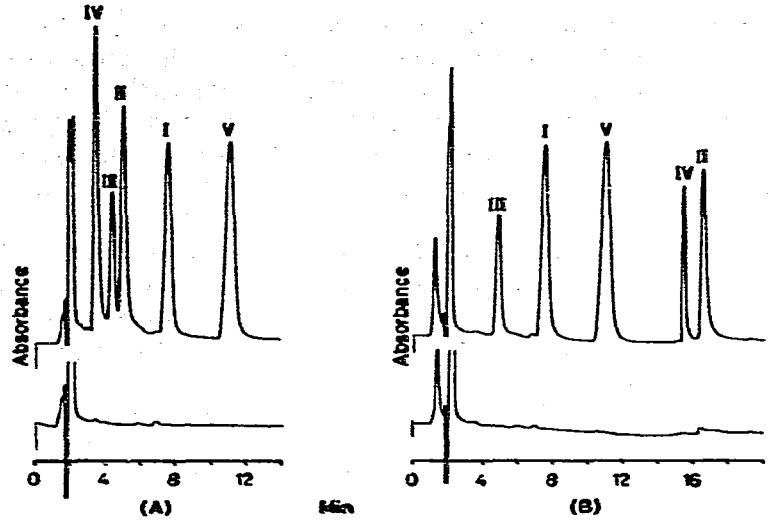

Fig. 3. Typical chromatograms. A, isocratic elution; B, gradient elution. The chromatograms represent plasma spiked with 0.65 μg/ml of I, 0.72 μg/ml of II, 1.29 μg/ml of III, 3.30 μg/ml of IV and 0.92 μg/ml of V (upper traces) and control plasma extracts (lower traces). Sensitivity, 0.005 a.u.f.s. and attenuation × 16.

TABLE IV

ELECTROLYTE CONCENTRATION EFFECT ON THE ELUTION OF COMPOUNDS I, II, III, IV AND V

V = 1.00 for all determinations.

Electrolyte concentration (*M*)	Retention relative to compound V			
	I	II	III	IV
0.0225	0.69	0.45	0.40	0.28
0.0180	0.69	0.46	0.40	0.29
0.0135	0.69	0.47	0.40	0.31
0.0101	0.69	0.48	0.40	0.33
0.0068	0.69	0.50	0.40	0.37
0.0045	0.69	0.52	0.40	0.43
0.0023	0.69	0.60	0.40	0.61
0.0011	0.69	0.69	0.41	1.01
0.0006	0.69	0.96	0.41	1.77
0.0000	0.69	7.93	0.40	*

*Value greater than 10.00, not determined.

Two distinct separatory mechanisms appeared to be operating in the gradient elution system. Compounds I, III and V were separated by reversed-phase partition whilst compounds II and IV seemed to be separated by an ion-exchange mechanism. The following observations supported this theory. The nature of the electrolyte was relatively unimportant. Sodium phosphate and sodium chloride produced separations almost identical to ammonium phosphate under the conditions described. Compounds II and IV eluted in a similar

manner from a cation-exchange column when subjected to the same gradient, but compounds I, III and V eluted with the solvent peak. The pK_a values of compounds II and IV were such that at the pH of the mobile phase, in the range 6.3 to 6.6, they would have been approximately 50% ionized. The pK_a values of compounds I, III and V were such that they would have been undissociated in this pH range and therefore unable to interact with any ion-exchange sites.

The two HPLC systems presented here had similar sensitivities for mebendazole and its metabolites. Of the two methods the isocratic elution system was favoured when the biological background levels were low and when adequate resolution existed between the metabolites. In cases where the biological background produced a tailing "solvent" peak which extended under the metabolites, the gradient system was found to be superior. The gradient system eluted the biological material more quickly than the isocratic system, provided increased separation of the metabolites and gave increased resolution of the minor metabolite (IV).

These systems are being used to estimate plasma levels of mebendazole and metabolites in patients suffering from hydatid disease. Fig. 4 presents typical chromatograms from one such patient.

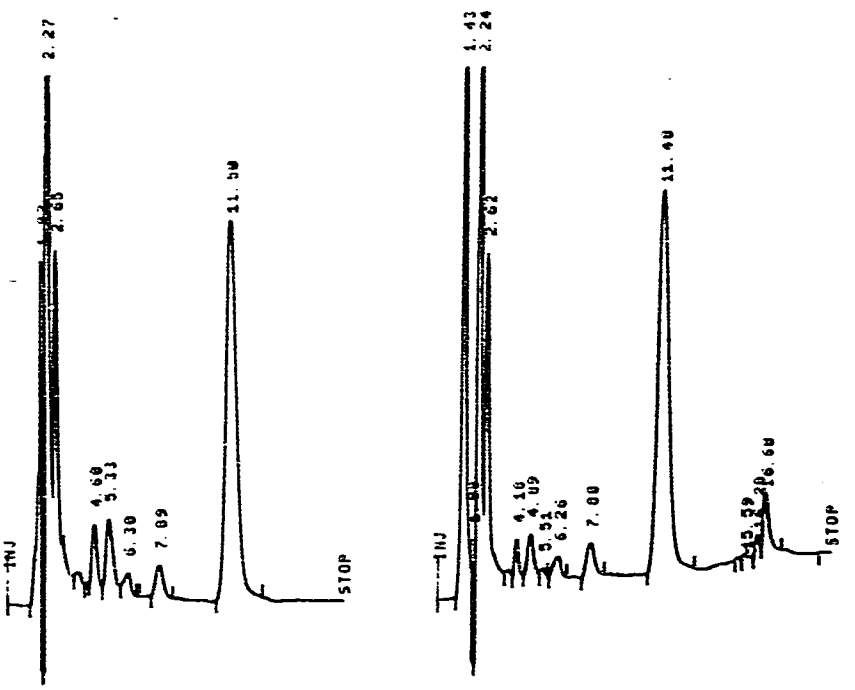

Fig. 4. Chromatograms from a steady-state patient representing 88 ng/ml of mebendazole (left, isocratic elution; right, gradient elution). Retention times: mebendazole, 7.89 min and 7.88 min; internal standard, 11.50 min and 11.48 min; sensitivity, 0.005 a.u.f.s. and attenuation × 8.

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