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Application of a high-performance liquid chromatography coulometric method for the estimation of mebendazole and its metabolites in human sera

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ABSTRACT

A novel, sensitive high-performance liquid chromatographic method, making use of coulometric detection, for the estimation of mebendazole and its metabolites in the sera of eight hydatidosis patients was devised. Recovery rates, precision, accuracy and sensitivity for each compound are reported and compared with those of the previously published methods.

INTRODUCTION

Mebendazole determination by high-performance liquid chromatography (HPLC) with UV detection has been widely reported and, according to the compounds to be determined, wavelengths of 254, 280, 300 and 315 nm have been employed [1–5]. The reported sensitivities with such detectors appear in each instance to be the highest possible, as these methods take into account the known difficulties associated with the low concentrations of drugs in biological fluids.

The use of amperometric electrochemical detectors has noticeably improved both the specificity and sensitivity of mebendazole determinations [6], although the application of a rather high voltage (+0.95 V) relative to an Ag/AgCl reference electrode to obtain a good sensitivity generates noise peaks.

This paper describes a new electrochemical determination method for mebendazole (I) and its known metabolites methyl-5-(α-hydroxybenzyl)benzimidazol-2-

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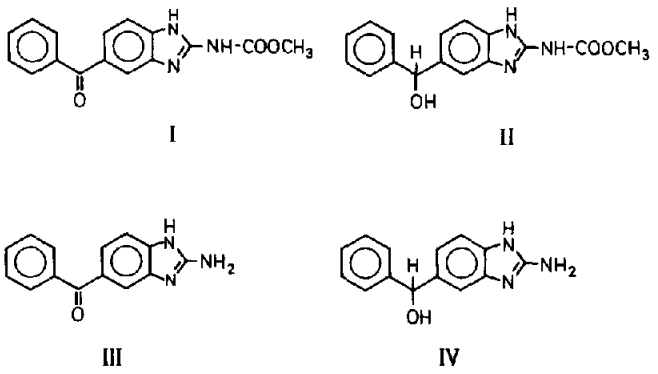

Fig. 1. Structures of mebendazole and its metabolites.

yl carbamate (II), 2-amino-5-benzoylbenzimidazole (III) and 2-amino-5-(α-hydroxybenzyl)benzimidazole (IV), in the sera of eight Italian patients (Fig. 1). These suffered from hydatid cysts of the parasitic tapeworm *Echinococcus granulosus*, and were therefore submitted to chemotherapy with 60 mg mebendazole per kg body weight per day.

Metabolites II and III were prepared according to published procedures with minor modifications; for metabolite IV a ned synthesis is presented (see Experimental).

EXPERIMENTAL

Materials and apparatus

Mebendazole (I) was supplied by Janssen (Beerse, Belgium); its metabolites II, III and IV were prepared as here described in analytically pure form; acetonitrile (HPLC grade) and all solvents and chemicals (reagent grade) were purchased from Carlo Erba (Milan, Italy).

Sep Pak C_{18} guard cartridges (Waters-Millipore, Milford, MA, U.S.A.) were used, and all HPLC solvents were obtained from distilled water treated with a Milli-Q system (Millipore).

The ^1H NMR spectra were recorded on a T-60 Varian spectrometer (Palo Alto, CA, U.S.A.) in hexadeuterodimethyl sulphoxide solutions with tetramethylsilane as internal standard. Abbreviations are as follows: bs = broad singlet; d = doublet; dd = doublet of doublets; m = multiplet; s = singlet. The figures in parentheses indicate the number of protons integrated.

Mass spectra were obtained with a Finnigan-Mat Model 5100 apparatus (San José, CA, U.S.A.), operated at 70 eV, by direct inlet probe of pure samples of compounds II, III and IV.

Melting points were determined both with a Büchi 530 capillary apparatus (Flawil, Switzerland) and with a Du Pont 910 differential scanning calorimeter (DSC) equipped with a 990 thermal analyser (Wilmington, DE, U.S.A.).

Microanalyses were performed in the Microanalysis Laboratory of the Istituto Superiore di Sanità.

Preparation of metabolites II, III and IV

Methyl-5-(α-hydroxybenzyl)benzimidazol-2-yl carbamate (II). This compound was prepared according to a published procedure [7] modified as follows. Mebendazole (5.0 g) was first dissolved with stirring in a heated mixture of dioxane (500 ml) and methanol (125 ml). The solution was allowed to cool to room temperature (15–20°C). Sodium borohydride (5.0 g) was added in 1.0-g portions over 8 h, and the mixture was stirred overnight at room temperature. The reaction was monitored by thin-layer chromatography (TLC) (silica gel plates; mobile phase, toluene–2-propanol–concentrated ammonia, 70:29:1, v/v) and extra sodium borohydride was added if necessary to complete the reduction: R_F of I *ca.* 0.6; R_F of II *ca.* 0.5.

The solvent was evaporated under reduced pressure at 35–40°C, and the resulting residue was suspended in water (100 ml), filtered and rinsed with water. The crude dried product weighed 5.0 g (99.4%); after crystallization from dimethyl sulphoxide–water, it melted at 323°C (capillary method). The reported m.p. was >300°C [7].

Analysis for $C_{16}H_{15}N_3O_3$: Found, H = 5.11%; C = 64.67%; N = 14.08%. Calculated, H = 5.09%; C = 64.63%; N = 14.14%.

^{1}H NMR data: chemical shift (δ, ppm): 7.43–6.83 (8), m, aromatics; 5.70 (2), s, –C*H*–O*H* (one exchangeable with trifluoroacetic acid); 3.70 (3), s, CH_3; 3.23 (*ca.* 1–2 H), very broad, –NH.

Mass spectrum (base peak underlined): 297 (M^+); 280 ($M^+ - 17$); 265 ($M^+ - 32$, methanol); 220 ($M^+ - 77$); 192 ($M^+ - 105$); <u>160</u> (265 − 105); 105 (benzoyl); 77 (phenyl); 59 ($COOCH_3$).

2-Amino-5-benzoyl-benzimidazole (III). This compound was prepared according to the literature [8] to give 97.9% yield of the crude product. Crystallization from water gave the pure product, m.p. 203–204°C (capillary) and 204°C (DSC). The values of the m.p. reported by various authors are: 193°C, no crystallization given [8]; 197–198°C, from ethanol–water [7]; 196–199°C, from methanol–water [9]. The reaction completeness was controlled by TLC under above conditions: R_F of the starting compound I *ca.* 0.6; R_F of product III *ca.* 0.3.

Analysis for $C_{14}H_{11}N_3O$: Found, H = 4.49%; C = 70.72%; N = 17.81%. Calculated, H = 4.67%; C = 70.87%; N = 17.71%.

^{1}H NMR data: chemical shift (δ, ppm): 7.55 (6), m, aromatics; 7.38 (1), dd, H-6; 7.15 (1), d, H-7; 6.58 (2), bs, NH_2; 6.00 (1), broad, –NH.

Mass spectrum (base peak underlined): 237 (M^+); <u>160</u> ($M^+ - 77$); 132 ($M^+ - 105$); 105 (benzoyl); 77 (phenyl).

2-Amino-5-(α-hydroxybenzyl)benzimidazole (IV). To a stirred solution of III (2.0 g) in methanol (100 ml), sodium borohydride (3 × 0.5 g) was added at room temperature over 3 h until hydrogen evolution had ceased (the reaction was

monitored by TLC under the above conditions: R_F of the starting compound III *ca.* 0.3; R_F of product IV *ca.* 0.2). The reaction mixture was then evaporated to dryness under reduced pressure, the residue was suspended in water and extracted with ethyl acetate, and the solvent evaporated. The yield was 1.8 g (89.3%), and the m.p. 187°C. After crystallization from water, the compound melted at 194–195°C (capillary) and at 194°C (DSC). The reported m.p. is 167–170°C [7]; the product was prepared by alkaline hydrolysis of II and obtained from ethanol–water as an oil that slowly crystallized.

Analysis for $C_{14}H_{13}N_3O$: Found, H = 5.41%; C = 70.32%; N = 17.66%. Calculated, H = 5.48%; C = 70.28%; N = 17.56%.

^{1}H NMR data: chemical shift (δ, ppm): 7.57–6.50 (8), m, aromatics; 6.10 (2) bs, $-NH_2$; 5.67 (1), s, C*H*–OH; 6.50–5.00 (*ca.* 1–2 H), very broad, –NH and/or –OH.

Mass spectrum (base peak underlined): 239 (M^+); 220 ($M^+ - H_2O - H^+$); 162 ($M^+ - 77$); <u>134</u> ($M^+ - 105$); 105 (benzoyl); 77 (phenyl).

Conditions for HPLC

The HPLC system consisted of a liquid chromatograph Series 4 from Perkin-Elmer (Norwalk, CT, U.S.A.) with a Rheodyne 7125 injection port (Berkeley, CA, U.S.A.), a 100 μl sample loop and a Coulochem 5100 A electrochemical detector (ESA, Bedford, MA, U.S.A.) equipped with a 5020 Model guard cell and a 5010 A Model analytical cell. The applied voltages were +0.70 V for the guard cell, +0.60 V for the first electrode and −0.10 V for the second electrode. The signal of the latter was sent to an LC-100 Perkin-Elmer laboratory computing integrator to calculate the obtained chromatogram peak heights. A 100 mm × 4.6 mm I.D. column (Shandon, Astmoor, U.K.) packed with the reversed-phase Hypersil SAS, particle size 5 μm, was employed. With this column the best results were obtained by modifying a previously described mobile phase [1]: a 0.01 *M* potassium dihydrogenphosphate solution containing 20% (v/v) acetonitrile adjusted to pH 3 with orthophosphoric acid. Elution was performed isocratically at room temperature at a flow-rate of 0.8 ml/min.

Calibration curve

The linearity of the instrumental response *versus* the amounts of the injected standard compounds was checked by injection of four different solutions of a mixture of standards I–IV (see Table I). Table I also lists the linear regression equations with their indexes of determination (r^2).

Compound I was dissolved in water–methanol–2 *M* formic acid (50:48:2, v/v); compounds II, III and IV were dissolved in 50% (v/v) aqueous methanol.

Preparation of samples

The sera were purified according to a published method [3]: 1 ml of serum was allowed to pass through a Sep-Pak C_{18} cartridge previously conditioned with 5

TABLE I

RESPONSE LINEARITY FOR DIFFERENT CONCENTRATIONS OF I–IV

y = peak height; x = injected amount.

Compound	Concentration range (ng/ml)	Regression equation	r^2	t_R (min)
I	0.24–0.94	$y = 88.99x - 2.97$	0.9945	7.8
II	0.21–0.84	$y = 44.92x + 1.35$	0.9574	4.5
III	0.16–0.64	$y = 121.58x - 0.78$	0.9938	5.5
IV	0.25–1.00	$y = 84.61x + 2.16$	0.9949	3.1

ml of methanol and 5 ml of 0.02 *M* potassium dihydrogenphosphate solution. The cartridge was first rinsed with 20 ml of water and 0.5 ml of 40% (v/v) aqueous methanol, then mebendazole and its metabolites were eluted with 2 ml of methanol. This eluate was evaporated to dryness under a nitrogen stream at room temperature, and the resulting residue was dissolved in 0.2 ml of methanol. An aliquot of this solution was injected into the column.

RESULTS

The assay reported employs isocratic reversed-phase HPLC, and the compounds are determined by coupled oxidation and reduction in a dual-electrode coulometric detector, the useful data for quantitative determination being given by the reduction electrode. Only substances that can be both oxidized and then reduced are measured: the products are well separated as is clearly shown in Fig. 2A. In Fig. 2B the serum chromatogram of a patient under treatment with mebendazole is presented.

The recovery of mebendazole (I) and its metabolites II, III and IV after complete pre-purification was determined by comparison with the results of the same analyses carried on a set of sera of healthy humans spiked with increasing amounts of mebendazole and its metabolites. As Table II shows, recovery rates were *ca.* 100%, and so no internal standard was used. The repeatability of the method, estimated from multiple intra-assays of a set of enriched sera, was satisfactory. Table II also lists standard deviations (S.D.) and coefficients of variation (C.V.).

The intra-assay precision and accuracy of instrumental responses for compounds I–IV were further verified by the addition of the standard compounds to a serum of healthy human subject. Aliquots thereof were enriched with increasing amounts of I–IV and then submitted to the whole analytical procedure. The resulting peak heights were plotted against the added amounts: Table III lists S.D., C.V. and accuracy values.

Peaks of compounds I–IV were identified by a combination of methods. Re-

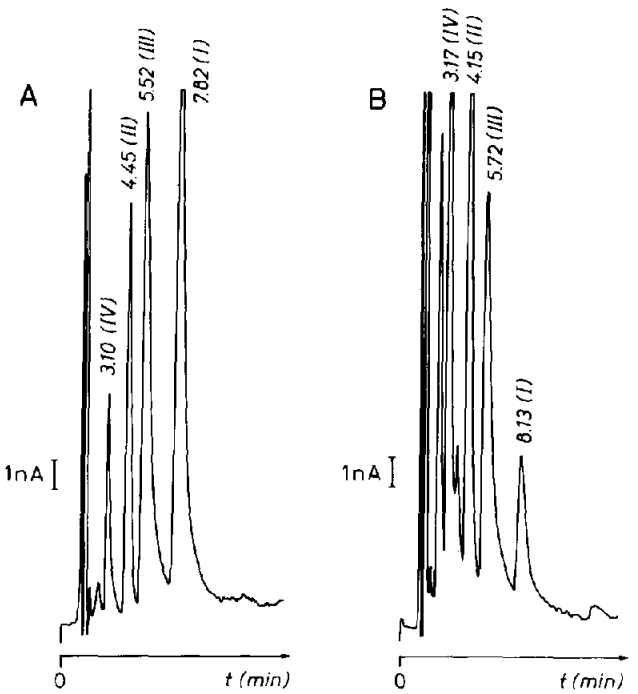

Fig. 2. (A) Chromatogram of a mixture of reference standards I–IV. (B) Chromatogram of serum from a patient under mebendazole treatment.

TABLE II

RECOVERY OF COMPOUNDS I–IV ADDED TO A SET OF HUMAN SERA, AFTER PURIFICATION AND CONCENTRATION

Compound	Concentration (ng/ml)	Recovery (%)	C.V. (%)
I	0.24	102.6 ± 1.7	1.7
	0.48	101.8 ± 1.4	1.4
	0.96	99.8 ± 1.4	1.4
II	0.21	99.2 ± 3.9	3.9
	0.42	105.4 ± 5.0	4.7
	0.84	96.6 ± 4.6	4.7
III	0.16	104.2 ± 3.2	3.1
	0.32	98.6 ± 2.9	2.9
	0.64	102.7 ± 2.6	2.5
IV	0.25	99.4 ± 3.1	3.1
	0.50	104.7 ± 2.9	2.8
	1.00	104.3 ± 2.7	2.6

TABLE III

INTRA-ASSAY PRECISION AND ACCURACY OF THE METHOD

Mean values (n = 10), standard deviations and coefficients of variation of analyses of a serum sample enriched with known amounts of I–IV.

Compound	Concentration calculated (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy[a] (%)
I	46.40	47.04 ± 0.32	0.68	+1.38
II	32.30	32.40 ± 0.34	1.05	+0.31
III	40.80	41.60 ± 0.10	0.24	+1.96
IV	49.00	50.40 ± 0.18	0.36	+2.86

[a] $\text{Accuracy (\%)} = \dfrac{\text{found mean concentration} - \text{calculated concentration}}{\text{calculated concentration}} \times 100.$

tention times and simultaneous injection of standard substances allowed all peaks to be identified; moreover, the different peaks gave specific voltammograms, which were obtained by repeated injections of a sample at various potentials while the peak heights were recorded *versus* the applied voltage. The scanning of voltammograms was accomplished in the two steps of oxidation and reduction. For oxidation potentials only electrode 1 was employed, whose signals at different voltages were recorded. For reduction potentials, electrode 1 was kept at a fixed potential on the plateau (+0.60 V), the voltage of electrode 2 was varied and its signals were recorded.

To make the comparison of voltammograms easier (samples and standards), it was found useful to divide, for a given injected amount of each compound, the height of the relevant peaks obtained at various potentials by the height of the peak resulting at the highest voltage. Oxidation and reduction voltammograms of compounds I–IV are reported in Fig. 3.

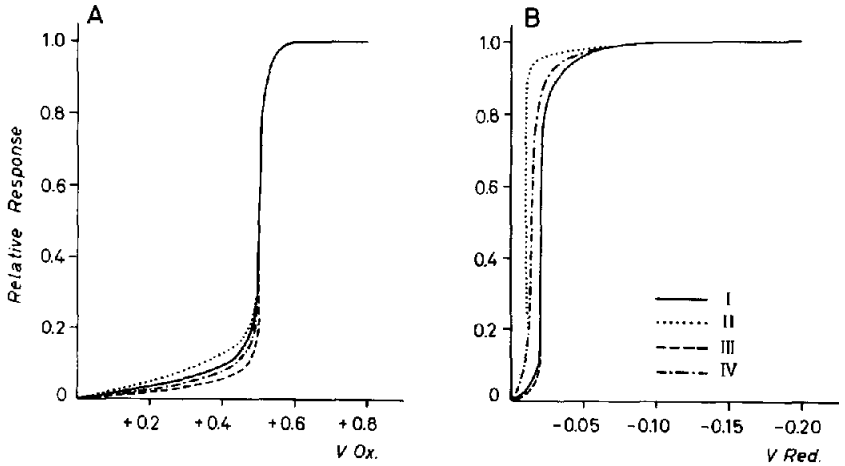

Fig. 3. (A) Oxidation voltammograms and (B) reduction voltammograms for compounds I–IV.

TABLE IV

LOWEST DETECTION LIMITS FOR COMPOUNDS I–IV

Detector	Injected volume (μl)	Injected compound (ng/ml)				Ref.
		I	II	III	IV	
Coulometric	5	0.25	0.40	0.17	0.20	This paper[a]
Amperometric	–	5.00	2.50	–	–	6[a]
UV, 254 nm	20	10.00	60.00	30.00	100.00	3[b]
UV, 313 nm	20	10.00	–	–	–	1[c]

[a] At a signal-to-noise ratio of 3.
[b] Based on S.D. $\leq 10\%$.
[c] Based on calibration curve.

The lowest limits of detection, at a signal-to-noise ratio of 3, were 0.25 ng/ml for I, 0.40 ng/ml for II, 0.17 ng/ml for III and 0.20 ng/ml for IV, a sensitivity noticeably higher than those reported previously (Table IV).

The present method was applied to the determination of mebendazole and its metabolites in the sera of eight hydatidosis patients of the Teramo district (Italy), who were submitted to oral mebendazole therapy with 60 mg/kg body weight per day.

The results corroborate the known variability [10] of the found levels of mebendazole and its metabolites in sera of patients under treatment. For the eight patients a total of 47 intra-assays were done. The numbers of patients and the relevant number of assays for each patient, given in order, were as follows: 1, 10; 2, 7; 2, 6; 2, 4; 1, 3. Higher levels were generally found for metabolites II and III, while only small amounts of IV and of unchanged I were detected.

The found figures (ng/ml of serum) ranged as follows: I, 3.3–69.5; II, 3.1–452.0; III, 5.4–156.3; IV, 1.9–24.2. For each assay ($n = 3$) a S.D. $\leq 10\%$ about the mean was observed.

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