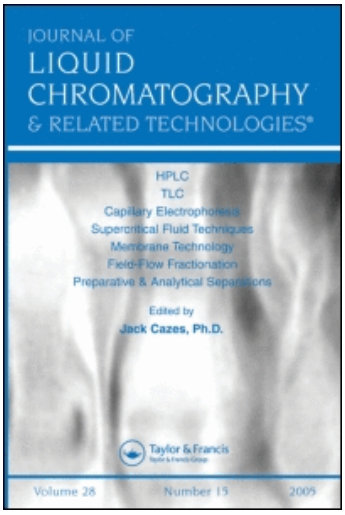

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A SELECTIVE AND SIMPLE RP-HPLC ASSAY TO QUANTIFY ALBENDAZOLE METABOLITES IN PLASMA

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ABSTRACT

A high performance liquid chromatographic (HPLC) method for the resolution of the two main metabolites of albendazole (ABZ): albendazole-sulphoxide (ABZ-SO) and albendazole-sulphone (ABZ-SO_2) is proposed and compared to other methods previously reported. Results are presented for the linearity, reproducibility, and sensitivity of these two metabolites. This method is useful in pharmacokinetic studies of ABZ and ABZ-SO. One example of the possible application of this HPLC assay method in pharmacokinetic studies is provided. ABZ-SO and ABZ-SO_2 were evaluated in plasma samples of mice after oral administration of a liquid formulation of ABZ-SO (50 mg/kg). During the 6 hours following oral administration to mice ABZ-SO concentrations were higher than ABZ-SO_2. Cmax for ABZ-SO was achieved in less than 60 minutes after oral administration.

INTRODUCTION

Albendazole (ABZ) is a benzimidazole of broad spectrum, used for therapy of human gastrointestinal helmintiasis and neurocysticercosis[1,2] as well as for the treatment of hydatid cists in veterinary.[3] Its metabolism has been studied in different animal species, which becomes complicated because of its many possible metabolites, including enantiomeric forms. The two main metabolites of albendazole (ABZ) are: albendazole-sulphoxide (ABZ-SO) and albendazole-sulphone ($ABZ\text{-}SO_2$).

Two kinds of HPLC methods have been previously reported: reverse-phase HPLC methods (RP-HPLC) and normal-phase HPLC methods (NP-HPLC).

Between the RP-HPLC methods (the most frequently used) we have made the following classification:

- Methods which require a previous extraction procedure of the samples.[4,5] These are complex procedures of extraction with organic solvents (for example, diethyl ether), followed by an evaporation step and posterior redissolution of ABZ metabolites. These are tedious processes that require special conditions for the manipulation of the samples. The precision of the analytical method depends on the percentage of recovery of the extraction step. Besides, the expenses in time and solvents advise their use only when extremely necessary.

- Methods that need fortified plasma samples.[4] Fortified samples of ABZ-SO are used for analytical determination when low concentrations of drugs in plasma are expected. Unfortunately, fortification of samples may induce some error due to the intrinsic variation for the method when the standard alone is determined for repeatability. This variation is multiplied by the error in the addition of a supposed constant amount of standard to the plasma samples. Besides, $ABZ\text{-}SO_2$ is expected in plasma too and its presence is not considered in this method.

- Methods that need cartridge conditioning[6] for sample preparation: Sep-Pack C_{18} cartridges are used for extraction of plasma samples in order to remove proteins. A careful preparation of the cartridge is required and the elution of the compounds decreases its concentration, so evaporation and redissolution steps are also needed to get acceptable drug concentration levels suitable for assay. Purification of the samples with the cartridge, does not improve the separation of ABZ-SO from plasma proteins as expected, so the efficacy for retention of plasma components by the cartridge may be questioned.

- Methods that require an acid mobile phase. In order to quantify benzimidazoles in tablets, powders or liquid formulations, an acid mobile phase (pH=3,2) has been used for a good resolution of the chromatographic peaks.[7] Previously, Gil-Grande et al.[8] have used a mobile phase of pH=3,0 for pharmacokinetic studies but capacity factor (K')for ABZ-SO was very low (0,85). Zeugin et al.[9] have reported a mobile phase containing a mixture of a phosphoric acid water solution with acetonitrile and methanol (75:18.7:6.3). Satisfactory results have been obtained with these methods and the usefulness of an acid medium to separate the two main metabolites of ABZ has been well established.[10,11]

Between the NP-HPLC methods, the most frequently used is the one described by Galtier et al.[12] An extraction with ethyl acetate and a mobile phase of 89% n-hexane and 11% ethanol are used. The conditions of this method were reproduced in our laboratory and we found some difficulty in the absorbance stabilization of the detector. Probably due to the high proportion and viscosity of n-hexane, which can also interfere at the low wavelength of detection (225 nm) proposed.

A modification of the extraction procedure and mobile phase reported by Zeugin et al.[9] is proposed in this paper to get a simple, economic, and reproducible method for ABZ metabolites separation and quantitative assay in plasma samples of mice.

EXPERIMENTAL

Materials

ABZ-SO and ABZ-SO_2 are supplied by Smith Kline Beecham (London, UK); HPLC grade methanol and phosphoric acid 85% analytical grade are purchased from Panreac (Madrid, Spain); HPLC grade acetonitrile is obtained from Merck (Darmstadt, Germany).

Instrumentation and Chromatographic Conditions

An HPLC system consisting of a Gilson 305 isocratic pump and a Gilson 231 XL automatic sampler equipped with an injection valve fitted to 100 µL-sampling loop is used. A constant flow of 1 mL/min is employed. The mobile phase was optimized by changing pH and organic solvent proportions. The best results were obtained with a mobile phase containing 800 mL of a water solution with 188 µL of

phosphoric acid 85% and 200 mL of acetonitrile. The final pH of the mobile phase can be adjusted to 2.5 with further addition of diluted phosphoric acid or KOH if necessary.

Analysis was performed on a Spherisorb ODS2 column (300 x 0,46 mm, 5 μm) from Teknokroma (Barcelona, Spain). The samples were measured at 290 nm using a Gilson 116 variable wavelength detector and data were recorded on a Spectra-Physics SP270 integrator (San Jose, Ca, USA).

Calibration of the assay was performed every twenty-four samples with three standards containing ABZ-SO and ABZ-SO_2 extracted in the same way as the samples, whose concentrations ranged from 1 μg/mL to 12 μg/mL (expected values).

Animals

Swiss CD-1 male mice (weight 30 g) were obtained from the Charles River (Spain), uniquely identified and housed individually in cages.

Sample Preparation

Two mL of methanol was added to aliquots of plasma (400 μL) to precipitate proteins. After vortex-mixing for 5 minutes, samples were centrifuged at 3000 g for 10 min and filtered through a Millipore Sterile Millex-GV 0.22 μm filter (Bedford, MA, USA). 100 μL-aliquots of the filtered fractions were injected into the HPLC system.

Treatment of Analytical Data

The resolution between two chromatographic peaks (R) was calculated from the equation:

$$R = \frac{2 \cdot (t_2 - t_1)}{w_1 + w_2}$$

Where t_1 and t_2 are the retention times and W_1 and W_2 the width of the peaks, measured by extrapolating the relatively straight sides to the baseline.

The slopes, intercepts, and linearity of each calibration curve were obtained by regression analysis. Confidence limits for intercept value (a) were calculated with the following equation:

$$a \pm t\, S_a$$

Where, t is the value of t-Student for n-2 degrees of freedom and a probability level of 0.05; S_a is the variance of the intercept value. If the zero value is between these two limits, the proportionally condition is achieved.

The confidence limits for the slope (b) of the regression line were calculated according to the following the expression:

$$b \pm t\, S_b$$

Where t is the value of t-Student for n-2 degrees of freedom and a probability level of 0.05; S_b is the variance of the slope. To evaluate the linearity of the method, the relative standard deviation for the slope is calculated according to the equation:

$$S_{b\,rel}(\%) = \frac{S_b}{b} \cdot 100$$

Detection limits (D.L.) are statistically calculated from the following equation:[13]

$$D.L. = S_0^2 \cdot \frac{\sqrt{n-2}}{n-1} \cdot \frac{t_p}{b}$$

Where n is the number of values, t_p is the value of t-Student for n-2 degrees of freedom at P= 0.05 level of significance, b is the slope and S_0^2 is the variance characterizing the dispersion of the experimental values with respect to the regression line. The limit for experimental detection is the lower concentration that can be found. The analytical recovery (%) was calculated from 100 x (amount found/amount added).

Stability of ABZ-SO and ABZ-SO_2 standards extracted from plasma was measured after storage at 4°C for 5 days. The slopes of the curves obtained were compared and were not statistically different ($P<0.01$), so the standards were stable for at least 5 days. The plasma samples were stable at -20°C for 1 year.

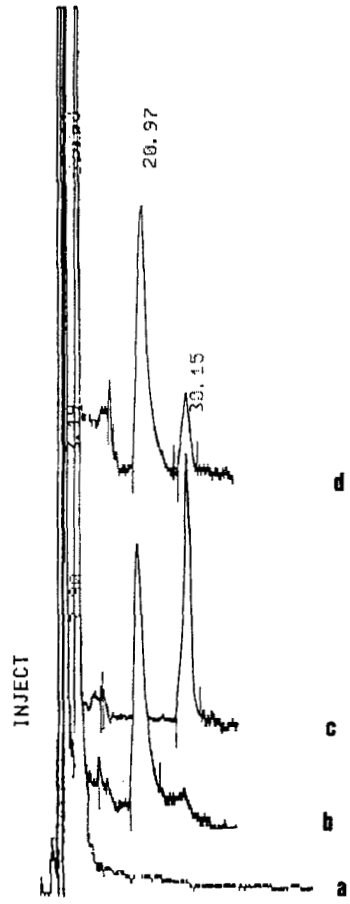

Figure 1. Chromatograms of blank plasma (a), ABZ-SO (b), ABZ-SO_2 (c) and a mixture of ABZ-SO and ABZ-SO_2 (d) in plasma samples.

Pharmacokinetic Study

ABZ-SO solution was orally administered by a single dose of 50 mg/kg to Swiss CD-1 mice. After drug administration, groups of three mice were sacrificed and blood samples were collected at different times (0.25, 0.75, 1.5, 2.0, 3.0, 4.5, and 6 h). Blood samples were heparinized and centrifuged individually after the extraction. Plasma samples were frozen until the HPLC analysis.

Table 1

Retention Time (t_a) and Capacity Factor (k') for Albendazole Sulphoxide and Albendazole Sulphone

Drug	t_a	k'
ABZ-SO	20.97	9.71
ABZ-SO_2	30.15	13.46

RESULTS

Chromatography

Chromatograms of blank plasma and plasma samples of the two metabolites are shown in Figure 1. Retention times and capacity factor for ABZ-SO and ABZ-SO_2 are presented in Table 1. Relative retention (a) and resolution (R) for the two peaks are 1.4 and 2.4 respectively.

Linearity

Standard curves were performed at concentrations of 1.01, 3.16, 6.32, and 12.62 μg/mL for ABZ-SO (r^2=0.997) and 1.02, 3.2, 6.4, and 12.8 μg/mL for ABZ-SO_2 (r^2=0.997). Each point was analyzed by triplicate and the curves and equations obtained are shown in Figures 2 and 3.

The slopes, intercepts, and linearity in terms of relative standard deviation of these calibration curves are summarized in Table 2.

Both calibration curves were linear in the ranges tested. The linearity for ABZ-SO_2 assay was better than for ABZ-SO as the standard deviation and regression coefficient values indicate. The intercept values of ABZ-SO and ABZ-SO_2 were not statistically ($P<0.05$) different from zero.

The concentration range and detection limit are summarized in Table 3. The detection limits evaluated by the statistical method were similar to those calculated according to the experimental method. The lowest detection limit calculated was obtained for ABZ-SO (0.52 μg mL^{-1}).

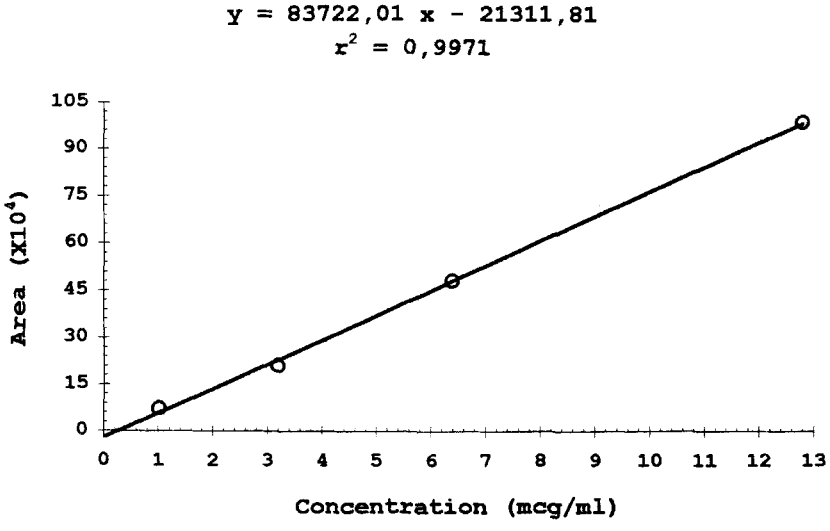

Figure 2. Standard calibration curves of ABZ-SO. Equation and regression coefficient.

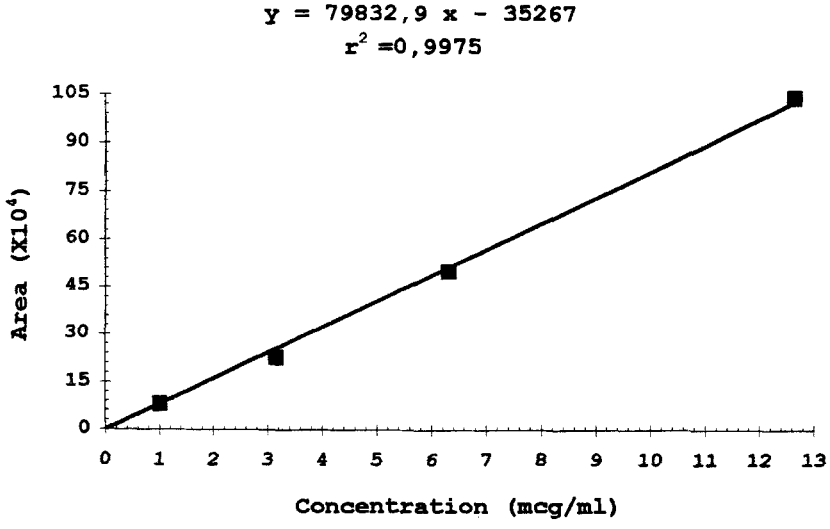

Figure 3. Standard calibration curves of ABZ-SO_2. Equation and regression coefficient.

Table 2

Comparative Analytical Data for the Determination of Albendazole Sulphoxide and Albendazole Sulphone

	ABZ-SO	ABZ-SO_2
Slopes bit S_b	83722.01±9534.62	77882.49±3375.56
Intercept Att S_a	21311.81±68556.55	23890.45±28437.65
Linearity S_{brel}(%)	5.17	1.97
r^2	0.9971	0.9975

Table 3
Concentration Range and Detection Limit for Albendazole Sulphoxide and Albendazole Sulphone

	ABZ-SO	ABZ-SO_2
Concentration Range (μ mL^{-1})	1-12.6	1-12.8
Detection Limit (μ mL^{-1}) Calculated:	0.52	0.58
Found:	0.64	0.65

Recovery

Standard curves were generated by the addition of known quantities of ABZ-SO and ABZ-SO_2 to a fixed amount of drug-free plasma until low, medium, and high concentrations ranging from 0.8 to 10.5 μg/mL were obtained and analyzed in the same way as drug-plasma samples. The extraction efficiency was determined by comparison of the obtained concentrations after injection with the known concentrations of fortified plasma samples of each analyte.

Table 4

Recovery of Albendazole Sulphoxide and Albendazole Sulphone from Plasma Samples (n=4)

Drug	Concentration (μg/mL)	% Recovery
ABZ-SO	0.84	106.69
	5.26	108.49
	10.53	100.52
$ABZ-SO_2$	0.85	101.13
	5.33	91.86
	10.66	89.46

Table 5

Inter-day Precision in Plasma Samples (n=3)

Drug	Concentration (μg/mL)	% RSD
ABZ-SO	1.0	3.6
	6.3	0.79
	12.6	0.92
$ABZ-SO_2$	1.0	6.7
	6.4	0.76
	12.8	0.94

The absolute recoveries in plasma samples at these low, medium, and high concentrations of ABZ-SO and $ABZ-SO_2$ were evaluated and they are shown in Table 4. The analytical recovery was between 100.52-108.49% for ABZ-SO and between 89.46-101.13% for $ABZ-SO_2$. The lowest recovery percentages for $ABZ-SO_2$ corresponded to the highest concentration (10.66 μg/mL), whereas at lower concentrations (0.85 and 5.33 μg/mL) better recovery percentages were obtained (91.86 and 101.13).

Specificity

Free plasma was collected and screened for interference at the retention times of ABZSO and $ABZSO_2$. Figure 1 shows a blank sample. No significant interference was observed.

Inter-Day Precision

Table 5 shows the mean results for ABZ-SO and $ABZSO_2$ when assayed during three consecutive days. For plasma concentrations (n=3) of 1, 6.3, and 12.6 μg/mL of ABZ-SO the method yielded relative standard deviations (RSD) of 3.60, 0.79, and 0.92% respectively. For equivalent plasma concentrations (1, 6.4, and 12.8 μg/mL and n=3) of ABZ-SO_2, the method yielded RSD of 6.70, 0.76, and 0.93%, respectively. Similar results were obtained by Torrado et al.[13] in inter-day precision of dl-α-Tocopherol acetate in dog plasma. Under the experimental conditions described the inter-day precision was better for ABZ-SO than ABZ-SO_2.

Application in a Pharmacokinetic Study

This analytical method has been used in a pharmacokinetic study in mice. Figure 4 represents the profiles of ABZ-SO and ABZ-SO_2 plasma concentrations versus time after oral administration of 50 mg/kg of an ABZ-SO solution to CD-1 male mice. Plasma concentrations are the mean values of at least three samples. Retention times for ABZ-SO and ABZ-SO_2 in plasma samples were 20.07 and 29.06 respectively, similar to the times reported in Figure 1. The peaks were well separated from plasma proteins and the resolution between them was acceptable.

Maximum plasma concentration (Cmax) for ABZ-SO (approximately 50 μg/mL) was reached at 30 minutes whereas ABZ-SO_2 (approximately 5 μg/mL) was reached later at 90 minutes. Delatour et al.[14] have reported maximum ABZ-SO plasma concentration in rats after oral administration of a suspension at 6 hours. These differences on Cmax can be due to differences between formulations (solution vs suspension) or between species (mice vs. rat).

Stability

ABZ-SO and ABZ-SO_2 in plasma were stable when stored at -20°C for up to 1 year. The stability of ABZ-SO and ABZ-SO_2 in methanol when stored at 4°C was stable for at least 3 days.

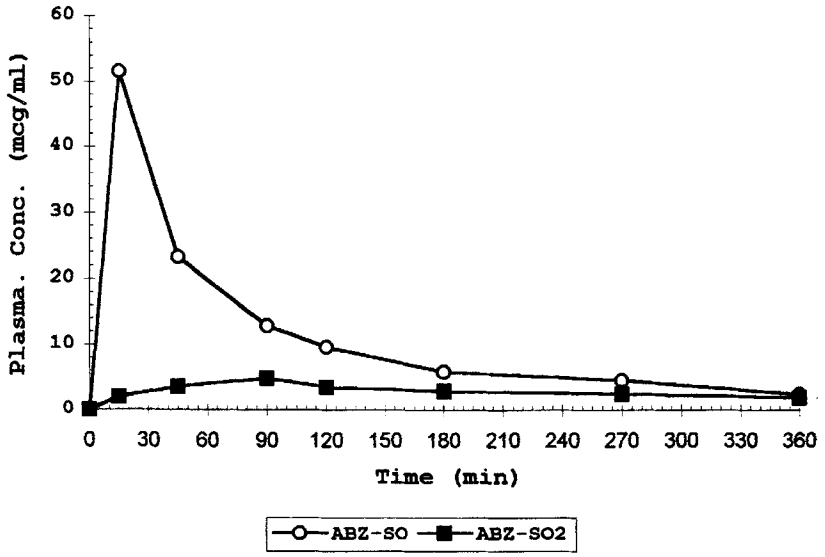

Figure 4. Representative plasma concentration-time profile for ABZ-SO and ABZ-SO_2 after an oral single dose 50 mg/kg of an ABZ-SO solution.

DISCUSSION

Compared to other chromatographic methods, this HPLC method has the advantages to be a simple and fast analytical procedure. Complete pharmacokinetic characterization of plasma samples with ABZ and its metabolites requires a sensitive and selective method.

The HPLC method reported here was useful for the analysis of ABZ-SO and ABZ-SO_2 in mice plasma. The major advantage of this method is the direct analysis without the necessity of a previous treatment of plasma samples.

Under the experimental conditions described here the ABZ-SO analysis was characterized by a linearity, detection limit, and recovery better than ABZ-SO_2 analysis. Inter-day precision, expressed as the relative standard deviation (RSD), was always below 7.0%.

For its selectivity, preliminary extraction steps are not necessary, and with better resolution than other methods previously reported, this HPLC method can be suitable for application in pharmacokinetic studies of ABZ and ABZ-SO.

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