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# Determination of albendazole and its main metabolites in ovine plasma by liquid chromatography with dialysis as an integrated sample preparation technique

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**Abstract**

Albendazole is a benzimidazole derivative with a broad-spectrum activity against human and animal helminth parasites. In order to determine the main pharmacokinetic parameters in sheep after oral and intravenous administration of a new formulation of albendazole (an aqueous solution), a fully automated method was developed for the determination of this drug and its main metabolites, albendazole sulfoxide (active metabolite) and sulfone in ovine plasma. This method involves dialysis as purification step, followed by enrichment of the dialysate on a precolumn and liquid chromatography (LC). All sample handling operations were executed automatically by means of an ASTED XL system. After conditioning of the trace enrichment column (TEC) packed with octadecyl silica with pH 6.0 phosphate buffer containing sodium azide, the plasma sample, in which a protein releasing reagent (1 *M* HCl) containing Triton X-100 was automatically added, was loaded in the donor channel and dialysed on a cellulose acetate membrane in the static-pulsed mode. The dialysis liquid consisted of pH 2.5 phosphate buffer. By rotation of a switching valve, the analytes were eluted from the TEC in the back-flush mode by the LC mobile phase and transferred to the analytical column, packed with octyl silica. The chromatographic separation was performed at 35°C and the analytes were monitored photometrically at 295 nm. Due to the differences in hydrophobic character between albendazole and its metabolites, a gradient elution was applied. The mobile phase consisted of a mixture of acetonitrile and pH 6.0 phosphate buffer. The proportion of organic modifier was increased from 10.0 to 50.1% in 12.30 min, then from 50.1 to 66.9% in 1.70 min. First, the gradient conditions and the temperature were optimised for the LC separation using the DryLab software. Then, the influence of some parameters of the dialysis process on analyte recovery was investigated. Finally, the method developed was validated. The mean recoveries for albendazole and its metabolites were about 70 and 65%, respectively. The limits of quantification for albendazole and its metabolites were 10 and 7.5 ng/ml, respectively. 

*Keywords:* Sample preparation; Dialysis; Gradient elution; Computer simulation; Albendazole; Benzimidazoles

## 1. Introduction

Albendazole (ABZ), [5-(propylthio)-1H-benzimidazol-2-yl]carbamate (cf. Fig. 1), is a broad-spectrum anthelminthic widely used in the veterinary

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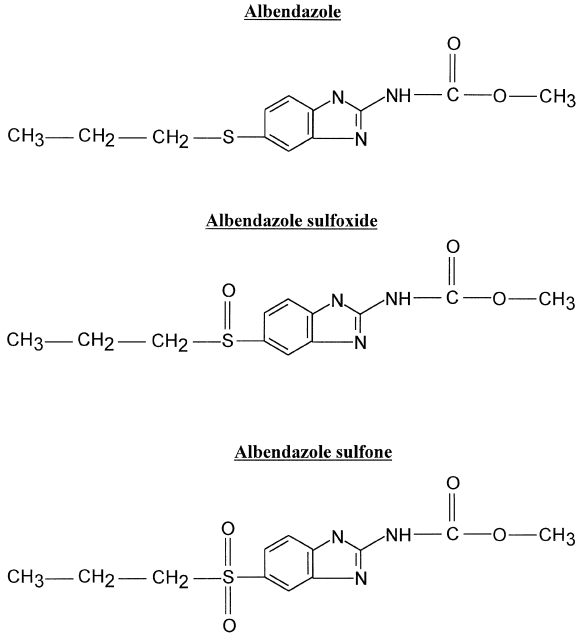


Fig. 1. Structures of albendazole and its two metabolites (albendazole sulfoxide and sulfone).

treatment of parasitic diseases and was recommended for human hydatid disease and other helminthiasis [1]. ABZ undergoes an extensive metabolism by liver microsomal enzymes to its major active metabolite, albendazole sulfoxide (ABZSO) (cf. Fig. 1) [2,3]. This metabolite is then metabolised to albendazole sulfone ($ABZSO_2$) (cf. Fig. 1), which does not appear to have any anthelminthic activity [2,3].

Several liquid chromatographic (LC) methods have been described for the determination of albendazole and/or its metabolites in biological fluids and especially in human plasma [2–6], but also in ovine [7,8] and mouse plasma [9]. The simultaneous determination of these three compounds has been only described in one of these methods [7]. However, the detectability of the latter was not sufficient: the limits of detection were 20, 50 and 40 ng/ml for ABZ, ABZ-SO and ABZ-$SO_2$, respectively.

In these methods, the analytes of interest were monitored photometrically at 225 [7], 254 [5], 290 [2,4,9] or 295 nm [6], but fluorometric detection was also achieved [3]. Prior to LC analysis, the sample preparation technique was mainly liquid–liquid extraction with an organic solvent [3,7] after deproteinisation [5] or acidification [4], possibly followed by a back-extraction in a basic medium [2]. A simple technique of protein precipitation [9] and solid-phase extraction [6] were also proposed. However, these off-line procedures may become tedious and time-consuming when a large number of samples have to be handled.

In this paper, a fully automated LC procedure is described for the simultaneous analysis of ABZ and its two metabolites (ABZ-SO and ABZ-$SO_2$) in ovine plasma and has been successively employed for the determination of the main pharmacokinetic parameters in sheep after oral and intravenous administration of a new formulation of albendazole (a solution) [10]. The very poor aqueous solubility and wettability of albendazole give rise to difficulties in the pharmaceutical formulation of oral or injectable solutions. Nevertheless, an aqueous solution of albendazole has been formulated owing to the good solubility of the complex obtained with hydroxypropyl-β-cyclodextrin.

The sample preparation technique proposed in this paper involves dialysis followed by the enrichment of the dialysate on a small pre-column packed with octadecyl silica and subsequent LC coupled to UV detection. All operations are executed automatically by means of a sample processor, the automated sequential trace enrichment of dialysates (ASTED) system. This technique has been shown to be useful for the determination of drugs in biological fluids and especially in plasma [11–23].

In the present study, the chromatographic conditions for the determination of albendazole and its main metabolites were first optimised. Due to differences of hydrophobic character between the drug and its metabolites, a gradient elution was applied. In order to optimise the separation under these conditions, a computer simulation was used by applying the DryLab software. Afterwards, the effect of some parameters of the dialysis process on analyte recovery has been studied. The influence of the pulse volume and the dialysis time as well as the pH of the dialysis liquid has been considered. The addition of a protein-releasing reagent to plasma samples has been more particularly investigated. These parameters have been optimised with respect to analyte recovery. Finally, the whole procedure has been validated and the results of the validation are presented.

## 2. Experimental

### 2.1. Chemical and reagents

Albendazole was supplied by Indis (Aartselaar, Belgium) and the metabolites (albendazole sulfoxide and sulfone) were synthesised in the Laboratory of Medicinal Chemistry at the University of Liège (Liège, Belgium) by controlled oxidation of albendazole by means of *m*-chloroperbenzoic acid. The purity of the products was checked by elementary analysis, by thin-layer chromatography and LC with UV detection. They were used without further purification. Potassium dihydrogenphosphate, potassium hydroxide, phosphoric acid (85%, w/w), chlorhydric acid (37%, w/v), Triton X-100 and sodium azide were purchased from Merck (Darmstadt, Germany). Monochloroacetic and trichloroacetic acids were supplied by Acros Chimica (Geel, Belgium) and *n*-octanoic acid was obtained from Sigma (St. Louis, MO, USA). All these reagents were of analytical grade. Methanol and far UV acetonitrile, both of LC grade, were purchased from Fisher Scientific (Leicestershire, UK). The water used in all experiments was purified by means of a Milli-Q system (Millipore, Bedford, MA, USA).

The analytical column was pre-packed with LiChrospher 60 RP Select B (particle size, 5 μm) (Merck). The trace enrichment column (TEC) containing octadecyl silica (Hypersil, particle size, 10 μm) was supplied by Gilson Medical Electronics (Villiers-le-Bel, France).

### 2.2. Apparatus

The ASTED system (Gilson) consisted of a XYZ auto-sampler equipped with two model 401C dilutors fitted with 1-ml syringes. The donor and acceptor channels (volumes: 370 and 650 μl, respectively) were separated by a cellulose acetate membrane (Cuprophan) with a molecular mass cut-off of 15 kDa. A Rheodyne model 7010 six-port switching valve (Berkeley, CA, USA) was fitted either with a 100-μl injection loop or with a TEC. The TEC (5.8×4.6 mm) consisted of a titanium tube contained within a stainless steel holder (Gilson).

The chromatographic system was composed of a model 305 pump coupled with a model 805 manometric module (Gilson) and of a model Dynamax UV-1 variable-wavelength UV–visible absorbance detector (Rainin, Woburn, MA, USA).

The separation was performed on a LiChroCart analytical column (125×4 mm I.D.) preceded by a LiChroCart guard column (4×4 mm I.D.) from Merck, both packed with the same stationary phase. They were thermostated at 35±0.1°C in a model 20 B/VC Julabo waterbath (Seelbach, Germany).

The control of the LC and ASTED systems were effected through a resident software Gilson 715 HPLC system controller loaded on an IBM compatible computer (PC-AT; CPU 80486) and the 722 keypad software, respectively.

The computer simulation was carried out with DryLab software for Windows, version 2.0 (LC Resources, Walnut Creek, CA, USA).

### 2.3. Chromatographic conditions

Unless otherwise stated, the mobile phase consisted of a mixture of acetonitrile and 0.05 *M* phosphate buffer, pH 6.0. The proportion of organic modifier was increased from 10.0 to 50.1% in 12.30 min, then from 50.1 to 66.9% in 1.70 min. Prior to use, the phosphate buffer was degassed for 15 min in an ultrasonic bath. The chromatographic separation was performed at 35°C using a constant flow-rate of 1.0 ml/min. The three analytes were monitored photometrically at 295 nm.

The 0.05 *M* phosphate buffer (pH 6.0) was prepared in a 1000-ml beaker by dissolving 6.8 g of potassium dihydrogenphosphate in 900 ml of water. The pH was adjusted to 6.0 with 1 *M* potassium hydroxide. The buffer solution was then transferred quantitatively to a 1000-ml volumetric flask and water was added to the mark. Before use, the phosphate buffer was passed through a 0.45-μm membrane filter from Schleicher and Schuell (Dassel, Germany).

### 2.4. Standard solutions

Stock solutions of ABZ, ABZ-SO and ABZ-$SO_2$ were prepared in methanol at a concentration of 0.1 mg/ml. Each standard solution was stored in a refrigerator at 4°C when not in use and were prepared once a month.

*2.4.1. Solutions used for method development*

During method development, an intermediate solution of ABZ was prepared by diluting 5.0 ml of the stock solution with a mixture of methanol–water (50:50, v/v) in order to obtain a concentration of 10 μg/ml. A mixed solution of ABZ-SO and ABZ-$SO_2$ (concentration, 10 μg/ml for each compound) was also prepared by applying the same procedure. These intermediate solutions were stored in a refrigerator at 4°C and were found to remain stable for at least 1 week.

These solutions were then diluted with water or plasma to reach a final concentration of about 500 ng/ml for each analyte. The latter solutions were prepared daily.

*2.4.2. Solutions used for method validation*

Three mixed solutions of ABZ, ABZ-SO and ABZ-$SO_2$ were prepared by diluting a stock solution of ABZ, ABZ-SO and ABZ-$SO_2$ with a mixture of methanol–water (50:50, v/v) in order to obtain concentrations of 10, 2 and 0.2 μg/ml. These diluted solutions were stored in a refrigerator at 4°C and were prepared daily. They were used to spike plasma samples (0.5 ml) in order to obtain calibration samples at concentrations of 5, 10, 20, 100, 500, 750 and 1000 ng/ml, as well as validation samples corresponding to the quality control samples used in routine analysis. The validation samples were prepared in pools with final concentrations of 10, 30, 500 and 850 ng/ml. Individual aliquots of 1 ml were then stored in polypropylene tubes at −20°C.

*2.5. Automated sample preparation*

After thawing of the plasma sample, the only manual operations were the centrifugation of the plasma sample at 3900 *g* for 10 min and the introduction of an aliquot (0.5 ml) into a polypropylene vial (0.85 ml) located in the appropriate rack of the sample processor. All other sample handling operations were then executed automatically by the ASTED system.

The automatic procedure started by the washing of the needle by 1.0 ml of a mixture of pH 2.5 phosphate buffer–methanol (80:20, v/v) (dispensing flow-rate, 6.0 ml/min). Between each step, the needle was also rinsed with 1.0 ml of the same solution (flow-rate, 30 ml/min) and an air-gap volume of 5 μl was generated inside the transfer tubing before pipetting the next liquid in order to avoid cross-contamination.

Unless stated otherwise, the automatic sample preparation was performed as follows:

- *TEC conditioning* (flow-rate, 2.0 ml/min): the TEC was conditioned with 1.0 ml of pH 6.0 phosphate buffer containing 0.005% (w/v) of sodium azide.
- *Addition of the protein-releasing reagent to the plasma sample:* 400 μl of plasma were introduced into a vial placed on the collector rack at a flow-rate of 1.0 ml/min. A 40-μl volume of 1 *M* HCl acid containing 0.1% of Triton X-100 was aspirated by the needle of the first dilutor at a flow-rate of 0.2 ml/min and then dispensed at the same flow-rate in the collector vial. Afterwards, the sample was homogenised by air bubbling (air volume, 0.3 ml; flow-rate, 0.5 ml/min).
- *Dialysis:* 185 μl of the sample were loaded in the donor channel of the dialyser at a flow-rate of 1.0 ml/min. During the dialysis process, the sample was kept static while the dialysis liquid consisting of pH 2.5 phosphate buffer was passed through the acceptor channel in 20 successive 0.325-ml pulses at a flow-rate of 1.0 ml/min. After dialysis, each pulse was dispensed onto the TEC at the same flow-rate.
- *TEC washing* (flow-rate, 1.0 ml/min): when dialysis in the static-pulsed mode was discontinued, the TEC was washed with 1.0 ml of pH 6.0 phosphate buffer.
- *Elution* (flow-rate, 1.0 ml/min): by rotation of a switching valve, the analytes were eluted from the TEC to the analytical column in the back-flush mode with the LC mobile phase.
- *Washing of the dialyser* (flow-rate, 3.0 ml/min): the donor channel was rinsed with 4.0 ml of a mixture of pH 2.5 phosphate buffer–methanol (80:20, v/v) containing 0.1% of Triton X-100, while the acceptor channel was washed with 4.0 ml of the dialysis liquid.

The chromatographic analysis of the prepared sample was then performed during the treatment of the next sample (concurrent mode).

The pH 2.5 phosphate buffer was prepared in a 1000-ml beaker by dissolving 6.8 g of potassium

dihydrogenphosphate in 900 ml of water. The pH was adjusted to 2.5 with diluted phosphoric acid (17%, w/w). The buffer solution was then transferred quantitatively to a 1000-ml volumetric flask and water was added to the mark. Before use, the phosphate buffer was passed through a 0.45-μm membrane filter from Schleicher and Schuell (Dassel). The pH 6.0 phosphate used during the dialysis process was the same as that of the LC mobile phase.

## 3. Results and discussion

### *3.1. Optimisation of the LC conditions*

Due to the differences of hydrophobic character between albendazole and its two metabolites, the simultaneous separation of three analytes under isocratic conditions would give rise to excessive run times. Moreover, albendazole, which is more hydrophobic than its metabolites, would elute very slowly so that the peak corresponding to this compound would be broadened to the point that its detection and its quantification would be more difficult. Therefore, a gradient elution was applied.

Recently it was found that simultaneous changes in gradient steepness and temperature represent a powerful way for optimising sample resolution and method selectivity [24,25]. In order to predict the separation of analytes as a function of temperature and gradient conditions, a software dedicated to the optimisation of chromatographic separations was used, namely the DryLab software. The used approach was based on four initial experiments, in which temperature and gradient time were allowed to vary, as indicated in Fig. 2. The specific values of temperature were 30 and 60°C, while the gradient times were 20 and 60 min. The proportion of organic modifier (acetonitrile) was increased from 5 to 100% according to a linear gradient. The buffer of the LC mobile phase was consisted of 0.05 *M* phosphate buffer, pH 6.0. Spiked plasma samples were analysed under these chromatographic conditions.

In each preliminary chromatogram, the peaks of interest corresponded to albendazole and its two metabolites. The retention times and the areas of these peaks were introduced into the software. However, in order to improve method selectivity, the

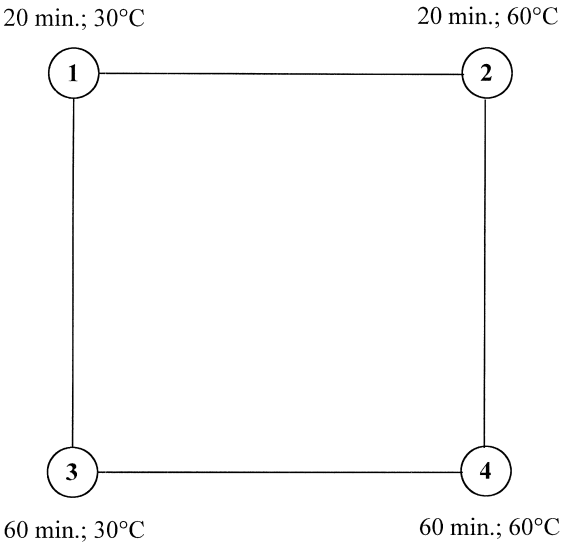


Fig. 2. Four experiments required for optimising temperature and gradient time.

retention times and areas of the peaks corresponding to the main endogenous components of plasma present under each operating condition were also entered into the software and were considered as potential interferences.

Following the entry of the whole data, a simulated chromatogram, as can be seen in Fig. 3A, was obtained under optimal conditions deduced from the software with an analysis time not exceeding 20 min. The optimal separation of the three compounds was obtained by simultaneously optimising gradient range, temperature and gradient time. The optimal conditions are presented in Fig. 3A: the temperature was settled at 35.4°C, the gradient time at 14 min and the gradient range was segmented. As shown in this figure, the peaks of interest were sufficiently resolved and separated from the endogenous components of plasma. Moreover, the experimental chromatogram (cf. Fig. 3B) obtained after the analysis of a spiked plasma sample was quite comparable to the simulated chromatogram, which demonstrates the very good prediction of retention times and resolution values obtained by applying this optimisation software. The comparison between this typical chromatogram and the chromatograms presented in Fig. 3C obtained after the analysis of a blank plasma sample under the optimal chromatographic and

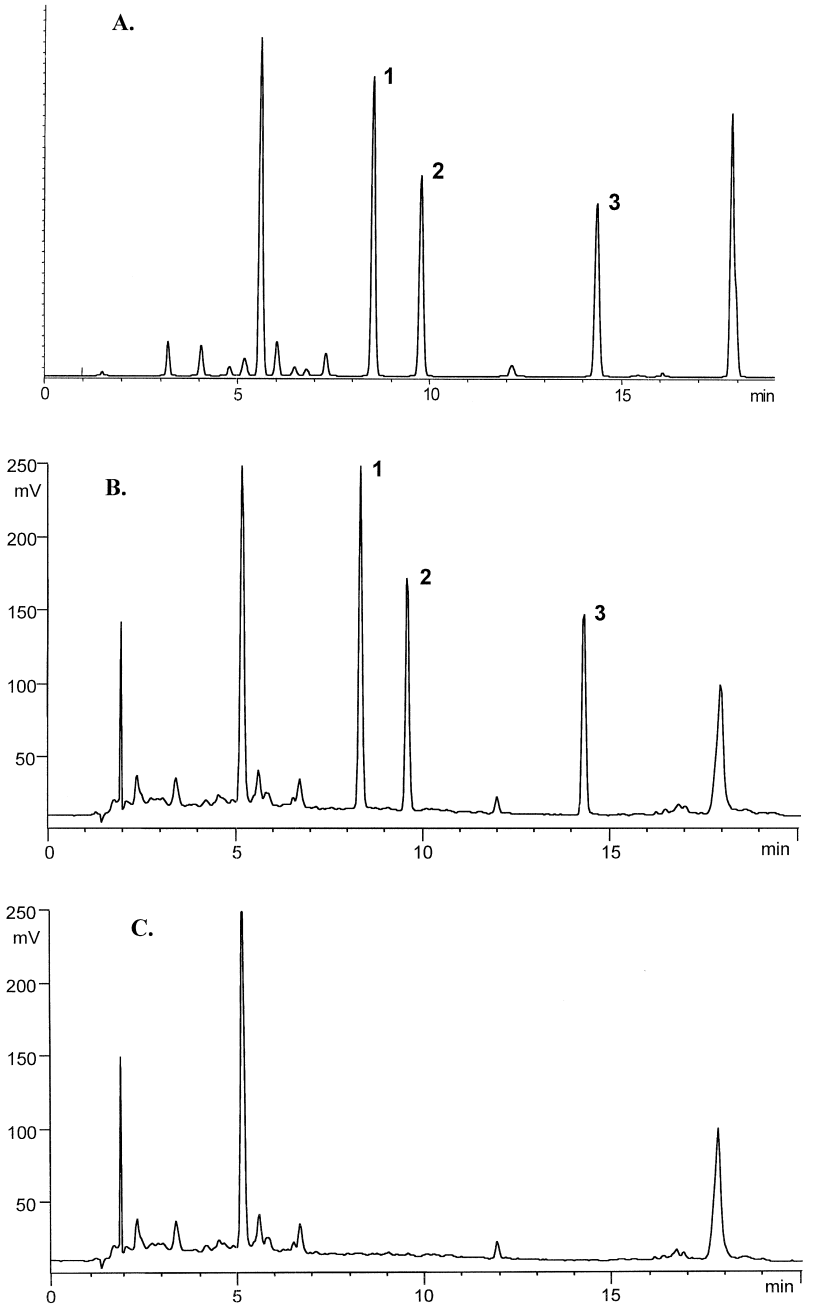


Fig. 3. Optimised separation of albendazole and its main metabolites in ovine plasma. (A) Simulated chromatogram. (B) Experimental chromatogram obtained from a spiked plasma sample (concentration, 500 ng/ml). (C) Typical chromatogram obtained from the analysis of a blank plasma sample. Gradient time, 14.0 min; temperature, 35.4°C; gradient range (segmented), 10–50.1% of acetonitrile in 12.30 min, then 50.1–66.9% of acetonitrile in 1.70 min. Other conditions as given in Section 2. Peaks: (1) albendazole sulfoxide; (2) albendazole sulfone; and (3) albendazole.

dialysis conditions also demonstrates the excellent selectivity of this method. Indeed, no interfering peaks from endogenous components of plasma were observed at the retention times of albendazole and its metabolites.

Compared to the previously published methods [2–6,8,9], the present method has the advantage to separate the three compounds of interest in the same chromatographic run due to the application of a gradient elution. Moreover, it is fully automated and it is the first time that an automated method has been described for the determination of this important anthelmintic agent and its two metabolites. The time for sample preparation and liquid chromatographic analysis was about 30 min. Consequently, 48 samples could be analysed every day.

### 3.2. Dialysis and trace enrichment

During the development of the method coupling dialysis and trace enrichment to LC, the objective was to obtain a high dialysis efficiency and a good method selectivity. On the basis of our expertise [17,18,23], several dialysis conditions have been settled a priori in order to increase analyte recovery: the choice of the static-pulsed dialysis mode, the selection of a relatively high volume of dialysis inferior to the breakthrough volume of the least retained analyte on the TEC and the selection of relatively low flow-rates for aspirating the dialysis liquid and dispensing the dialysate. Under such as conditions, the concentration gradient over the membrane remains high and the diffusion of the analytes through the membrane is more important. The time of contact of the sample with the membrane is also higher.

#### 3.2.1. Influence of the pulse volume and dialysis time on analyte recovery

In the two experiments presented in Table 1, the time of contact of the sample with the membrane did not change [23] but, as can be seen in this table, the recoveries for the metabolites of albendazole increased by using a pulse volume lower than the volume of the acceptor channel. This decrease of volume shows an increase of the residence time of each pulse in the acceptor channel. Consequently, the dialysis time increased globally, which allowed a better diffusion of the analytes through the membrane. These results also show that the dialysis time is a more important parameter to be considered than the dialysis volume. Indeed, the relative recoveries were higher in this first experiment, even when the dialysis volume was lower.

Table 1
Influence of the pulse volume on the recovery of albendazole metabolites[a]

| Experiment | Volume of pulses (μl) | Dialysis volume (ml) | Relative recovery±SD (%; $n=2$) | |
|---|---|---|---|---|
| | | | Albendazole sulfoxide | Albendazole sulfone |
| 1 | 325 | 6.5 | 72.6±0.2 | 73.0±0.5 |
| 2 | 650 | 13.0 | 68.3±0.3 | 67.6±0.3 |

[a] Dialysis liquid, pH 6.0 phosphate buffer; aspirating and dispensing flow-rates, 1.0 ml/min (exp. 1), 2.0 ml/min (exp. 2); Sample, aqueous solution. Other conditions as given in Section 2.

#### 3.2.2. Effect of the pH of the dialysis liquid on analyte recovery

For ionised or ionisable compounds, the pH of the dialysis liquid may have a significant influence on analyte recovery. The results presented in Table 2 confirm the effect of the dialysis liquid pH on recoveries for the metabolites of albendazole. Analyte recovery increased when the pH of the dialysis liquid decreased. These results indicate that probably a stronger hydrophobic interaction takes place between the analytes and the membrane at higher pH. ABZ-SO and ABZ-$SO_2$ are basic compounds and are thus less ionised at pH 6.0 than pH 2.5. Consequently, it is possible the hydrophobic binding of these analytes to the membrane would be stronger at a higher pH, which could decrease analyte recovery.

Table 2
Influence of the pH of the dialysis liquid on analyte recovery[a]

| pH of the dialysis liquid | Relative recovery±SD (%; $n=2$) | |
|---|---|---|
| | Albendazole sulfoxide | Albendazole sulfone |
| 6.0 | 68.3±0.3 | 67.6±0.3 |
| 2.5 | 73.1±1.3 | 72.4±1.3 |

[a] Dialysis liquid, phosphate buffer; aspirating and dispensing flow-rates, 2.0 ml/min; dialysis volume, 13 ml; volume of pulses, 650 μl; sample, aqueous solution. Other conditions as given in Section 2.

### 3.2.3. Influence of the addition of the protein releasing reagent to the plasma sample on analyte recovery

As can be seen in Table 3, the relative recoveries for the three compounds and especially for albendazole were lower in plasma than those observed with aqueous solutions of analytes. This decrease in recovery is usually attributed to protein binding of the analyte. Indeed, only the unbound drug fraction can actually diffuse through the membrane. Since the determination of the total drug fraction is needed, a protein releasing reagent, such as monochloroacetic acid (MCA), trichloroacetic acid (TCA) or chlorhydric acid, was added to the plasma sample prior to dialysis process. With these agents, the structure of proteins was modified by a change in the sample pH, which increased the concentration of the free analyte. A volume of 50 μl of 0.5 *M* MCA was sufficient to obtain recoveries for the metabolites of albendazole in plasma comparable to those observed with aqueous solutions. On the other hand, the recovery for albendazole was still too low. The addition of 0.1% (v/v) of Triton X-100, a non-ionic surfactant, to the MCA solution caused a slight increase in recovery for albendazole, which demonstrated a strong interaction of this compound with the membrane. TCA did not give rise to better results than MCA with respect to analyte recovery. Another approach used to increase the free concentration of a drug before the application of dialysis was the use of a compound, such as *n*-octanoic acid, which could displace the compound of interest from its binding sites. This displacement can be competitive (competition for a common binding site) or non-competitive (the displacer alters the tertiary structure of the protein and induces the release of the compound) [13]. As shown in Table 3, the recovery of albendazole increased significantly when 100 μl of 10 m*M* *n*-octanoic acid containing Triton X-100 were added to ovine plasma. Nevertheless, the best results with respect to analyte recovery were obtained by using 1 *M* HCl containing 0.1% (v/v) of the non-ionic surfactant as protein releasing reagent. Indeed, the recoveries for albendazole and its metabolites were significantly the same in plasma as in aqueous solutions.

### 3.2.4. Removal of a 'memory' effect for albendazole

After the analysis of a plasma sample containing a relatively high concentration of albendazole, a residual analyte peak was observed on the blank chromatogram during method development. This 'memory' effect was certainly due to the strong

Table 3
Influence of the addition of a protein releasing reagent to plasma on analyte recovery[a]

| Sample | Protein releasing reagent | Volume (μl) | Relative recovery±SD (%; $n=2$) | | |
|---|---|---|---|---|---|
| | | | Albendazole sulfoxide | Albendazole sulfone | Albendazole |
| Aqueous | – | – | 71.4±0.2 | 70.0±0.6 | 66.5±0.7 |
| Plasma | – | – | 67.5±2.5 | 63.0±2.5 | 49.3±1.0 |
| Plasma | 0.5 *M* MCA | 50 | 71.0±0.4 | 67.0±1.5 | 49.5±0.8 |
| Plasma | 0.5 *M* MCA | 100 | ND | ND | 49.9±1.0 |
| Plasma | 0.5 *M* MCA+0.01% of Triton X-100 | 100 | ND | ND | 50.0±0.2 |
| Plasma | 0.5 *M* MCA+0.1% of Triton X-100 | 100 | ND | ND | 55.0±0.5 |
| Plasma | 0.5 *M* TCA | 50 | ND | ND | 47.8±0.1 |
| Plasma | 0.5 *M* TCA | 100 | ND | ND | 51.0±2.4 |
| Plasma | 1 m*M* *n*-octanoic acid+0.1% of Triton X-100 | 100 | ND | ND | 49.4±0.8 |
| Plasma | 10 m*M* *n*-octanoic acid+0.1% of Triton X-100 | 100 | ND | ND | 61.9±1.0 |
| Plasma | 1 *M* HCl+0.1% of Triton X-100 | 50 | 71.2±0.3 | 67.2±0.9 | 66.6±1.3 |

[a] Volume of plasma, 500 μl. Other conditions as given in Section 2. ND, not determined.

interaction of albendazole with the membrane. Since a poor precision is often obtained on successive analyses in the presence of 'memory' effects, it is needed to eliminate these effects. As shown in Fig. 4A, the volume of the washing liquid of the dialyser constituted of a mixture of pH 2.5 phosphate buffer–methanol (80:20, v/v) containing 0.1% of Triton X-100 was first increased. Under these conditions, the memory effect decreased, but remained present. It was completely removed by increasing the volume of the 0.5 *M* MCA containing 0.01% of the non-ionic surfactant, which was added to plasma in order to disrupt drug–protein binding (cf. Fig. 4B).

### *3.3. Method validation*

The method was then validated by applying a new strategy proposed by a Commission of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) for the validation of bioanalytical methods [26,27]. The results are presented in Table 4. The validation protocol comprises two steps: a prevalidation step and a validation step itself. This strategy considers the validation criteria commonly used in bioanalysis, but focuses on the analysis of the response function as well as on the way of estimating and confirming the limit of quantitation and the concentration range and determining method accuracy. This strategy has been successfully applied for the validation of several bioanalytical methods [28,29].

#### *3.3.1. Evaluation of method selectivity towards endogenous components*

Fig. 5A–G shows typical chromatograms obtained after the analysis of a plasma sample spiked with the three compounds of interest at a concentration of 100 ng/ml and six independent blank plasma samples. The absence of interfering endogenous components at the retention times of albendazole and its main metabolites is clearly demonstrated in the figure.

#### *3.3.2. Analysis of the response function*

The analysis of the response function was performed in order to select the best calibration curve model. The selected concentration ranges were comprised between 5 and 1000 ng/ml for ABZ and between 5 and 750 ng/ml for ABZ-SO and ABZ-$SO_2$. The peak areas were taken as the analytical responses. Since the variances of the responses were not homogeneous at the 5% significance level by applying the statistical Cochran's and Levene's tests [26,27], a simple regression model using the least-squares method could not be applied. In order to describe the relationship between concentration ($x$) and response ($y$), the most appropriate regression model was obtained by performing a 'square root' transformation of the data ($\sqrt{x}-\sqrt{y}$) before the application of the least-squares method. With this mathematical transformation, the variances were found to be homogeneous at the 5% level. Moreover, the adequacy of the linear model was confirmed by a lack of fit test.

#### *3.3.3. Limit of quantification and determination of the concentration range*

As can be seen in Table 4, the limits of quantitation (LOQs) were equal to 10 ng/ml for albendazole and 7.5 ng/ml for its two metabolites, respectively. The detectability of this method was improved compared to that of previously published LC methods based on UV detection for the determination of albendazole and/or its metabolites in plasma [2,4–7,9].

These values were obtained from an accuracy profile by plotting as a function of the analyte concentration the mean recoveries (%) obtained for the responses at each calibration level as well as their one-sided confidence limits at the 95% level. The estimation of the standard deviation for intermediate precision was used to calculate these confidence limits. The LOQ corresponds to the concentration for which the confidence limits of the recovery are equal to 120 or 80%. An example of accuracy profile is presented in Fig. 6. Moreover, for each compound of interest, the lowest concentration level (5 ng/ml) had to be eliminated from the range since the confidence limits exceeded the norms of 80 and/or 120%. Consequently, the calibration ranges were restricted and are indicated in Table 4. As the calibration range had changed, an analysis of the response function was again performed. Under these conditions, the selected regression model was again a linear model with data transformation (square roots). The values for the slope, the intercept and their standard devia-

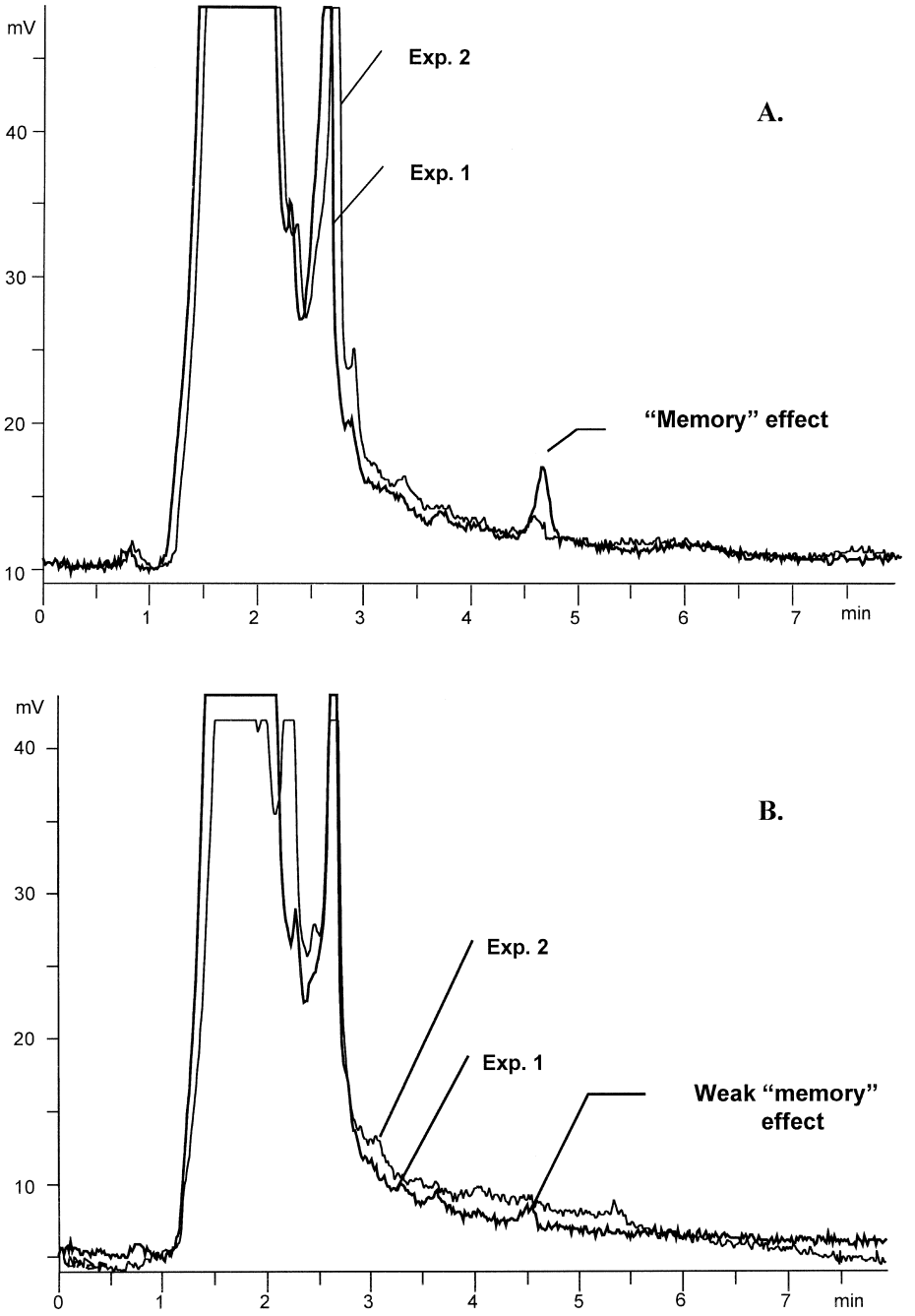


Fig. 4. Elimination of 'memory' effects for albendazole. (A) Decrease of the 'memory' effect. Volume of the washing liquid of the donor and acceptor channels of the dialyser, 2.0 ml (experiment 1), 4.0 ml (experiment 2). (B) Removal of the 'memory' effect. Volume of the protein releasing reagent solution added to 500 μl of ovine plasma, 50 μl (experiment 1), 100 μl (experiment 2); LC conditions (isocratic mode), pH 6.0 phosphate buffer–acetonitrile (40:60, v/v); protein releasing reagent, 0.5 *M* monochloroacetic acid containing 0.01% of Triton X-100; washing liquid, pH 2.5 phosphate buffer–methanol (80:20, v/v) containing 0.1% of Triton X-100.

Table 4
Results of method validation

| Validation criteria | Albendazole | Albendazole sulfoxide | Albendazole sulfone |
|---|---|---|---|
| Absolute recovery (%, mean±SD) | 69.9±4.4 | 65.5±5.7 | 65.3±2.8 |
| Calibration range (ng/ml) | 10–1000 | 7.5–750 | 7.5–800 |
| *Analysis of the response function:* Application of a linear regression model after transformation of data (square roots) | | | |
| Regression line ($n=45$): | | | |
| Slope±SD | 59.46±1.338 | 73.49±2.078 | 60.53±1.511 |
| Intercept±SD | −24.55±17.01 | −47.54±18.16 | −19.99±18.60 |
| $s_{y/x}$ | 23.17 | 17.22 | 16.30 |
| Lack of fit test ($F_{calc}/F_{(0.01;\ 9,30)}$) | 1.77/3.07 | 3.02/3.07 | 1.63/3.07 |
| Limit of quantitation (LOQ) (ng/ml) | 10 | 7.5 | 7.5 |
| Limit of detection (LOD) (ng/ml) | 2 | 1.7 | 1.4 |
| *Precision:* Repeatability/intermediate precision (RSD,%; $n=4$ replicates×3 days) | | | |
| LOQ | 4.7/6.9 | 6.2/8.0 | 4.8/5.4 |
| 3×LOQ | 2.9/4.4 | 2.8/6.8 | 2.3/2.8 |
| Medium concentration | 1.4/7.6 | 1.0/7.8 | 1.6/6.4 |
| 85% of the highest concentration | 0.8/3.4 | 0.6/1.6 | 0.4/0.4 |
| *Accuracy:* Recovery LCL/recovery UCL (%; $n=4$ replicates×3 days) | | | |
| LOQ | 88.0/113.5 | 87.1/117.3 | 94.2/114.9 |
| 3×LOQ | 99.8/117.5 | 92.6/118.8 | 103.0/114.0 |
| Medium concentration | 88.8/117.4 | 86.3/115.0 | 87.4/110.6 |
| 85% of the highest concentration | 90.2/102.3 | 91.0/96.5 | 91.9/93.2 |

tion as well as the residual standard deviation ($s_{y/x}$) are given in Table 4.

Moreover, the adequacy of the model was confirmed (cf. Table 4), the $F$ values obtained from the lack of fit test being lower than the critical value of the Table at the 1% level ($F_{(0.99;\ 9,30)}=3.07$) with the corresponding degrees of freedom.

### *3.3.4. Limit of detection*

The limit of detection (LOD) was estimated by applying the statistical method established by Miller and Miller [30]. As can be seen in Table 4, the LOD for the three compounds was found to be close to 2 ng/ml.

### *3.3.5. Determination of the extraction efficiency*

The analyte recoveries were determined at four different concentrations ranging from 20 to 1000 ng/ml for albendazole and from 20 to 750 ng/ml for its metabolites. The mean recoveries and their standard deviation ($n=12$) are presented in Table 4 and the results obtained show that the extraction efficiency was relatively constant over the range considered. The recoveries were calculated by comparing the peak areas obtained for the three analytes from freshly prepared spiked plasma samples with those found after the direct introduction on the TEC of aqueous standard solutions at the same concentrations. Moreover, a comparison of the peak areas obtained after direct introduction on the TEC of aqueous standard solutions with those found by direct injection of the same solutions using an injection loop of 100 μl instead of the TEC demonstrated that each analyte was entirely eluted from the TEC.

### *3.3.6. Determination of precision and accuracy*

Method precision and accuracy were determined

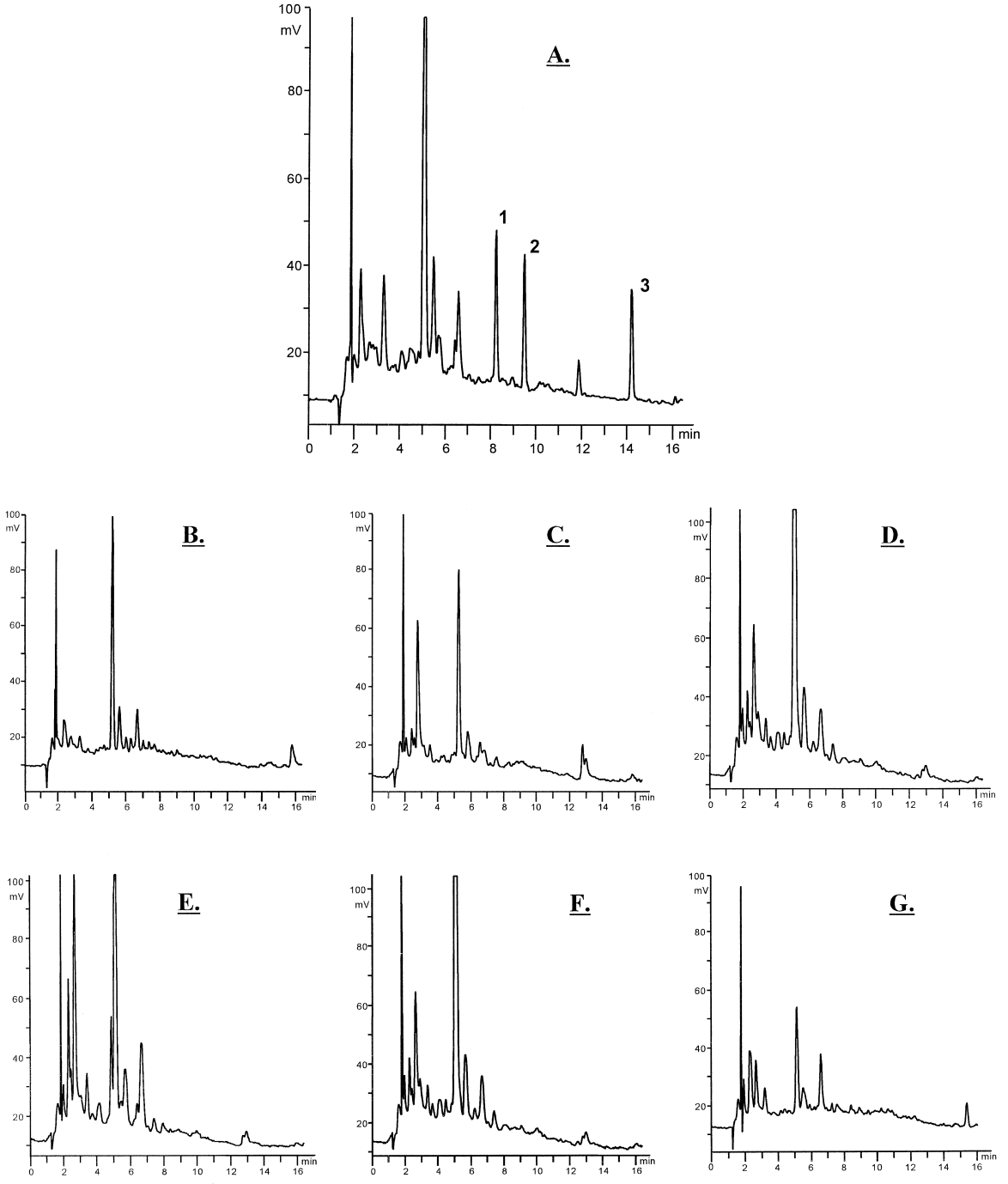


Fig. 5. Typical chromatograms obtained by on-line coupling of dialysis and trace enrichment to LC. (A) Chromatogram of a plasma sample spiked with the three analytes of interest (100 ng/ml). (B–G) Chromatograms obtained from six different blank plasma samples. Chromatographic and dialysis conditions as given in Section 2. Peaks: (1) albendazole sulfoxide; (2) albendazole sulfone; and (3) albendazole.

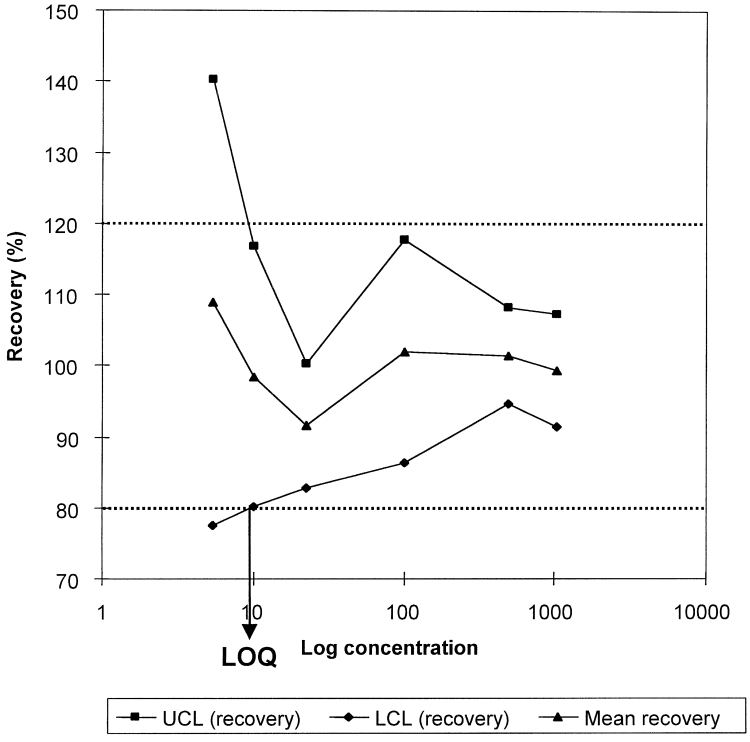


Fig. 6. Accuracy profile for the estimation of the limit of quantification and for the determination of the calibration range (example of albendazole).

from the analysis of validation samples corresponding to the quality control (QC) samples used in routine analysis. Four concentration levels representing the entire range investigated were selected, as can be seen in Table 4. Each validation sample was analysed four times for three consecutive days. This Table gives the relative standard deviations (RSDs) for repeatability and for time-different intermediate precision obtained at each concentration level. Except at the LOQ, the RSD values for repeatability were less than 3%. Moreover, irrespective the concentration level, the RSD values for intermediate precision did not exceed 8%. These results clearly demonstrate the good method precision.

Then, at each concentration level of the validation samples, the one-sided confidence limits of the mean recoveries (%) at the 95% level were computed by introducing the estimation of the standard deviation for intermediate precision. As shown in Table 4, the method is accurate, since the different confidence limits of the mean recoveries do not exceed the norms of 80 and 120%, irrespective the concentration level [26,27]. Moreover, the LOQ values estimated to be equal to 10 and 7.5 ng/ml for albendazole and its two metabolites, respectively, is confirmed, since precision and accuracy are also assessed at these concentration levels.

### *3.4. Application of the automated method*

This method was then applied successfully for the determination of pharmacokinetic parameters in sheep after oral and intravenous administration of a new formulation of albendazole (a solution).

After oral administration, albendazole was not detected at any time, while the metabolites (ABZ-SO and ABZ-$SO_2$) appeared relatively rapidly in ovine plasma, which confirmed the results of another study [8]. The peak concentration of the active metabolite, ABZ-SO, observed with the new formulation was significantly higher than that obtained with the

suspension and the bioavailability was also better [10].

After intravenous administration, the parent drug was detected in the plasma samples. However, the ABZ concentrations decreased rapidly, while the two metabolites appeared in the earliest plasma samples. These results confirm that albendazole is extensively metabolised in sheep. Detailed pharmacokinetic analysis after intravenous administration of the albendazole solution will be published later.

In the framework of these bioavailability and bioequivalence studies in sheep, the method proposed in the present study has been applied successfully to more than 200 plasma samples and has proved to be appropriate for the determination of albendazole and its two main metabolites in ovine plasma.

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