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Fully automated determination of sulfamethazine in ovine plasma using solid-phase extraction on disposable cartridges and liquid chromatography

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ABSTRACT

An automatic sample preparation procedure followed by on-line injection of the sample extract into a HPLC system has been developed for the quantitative analysis of sulfamethazine and its N_4-acetyl metabolite in ovine plasma. The sample clean-up was performed by solid-phase extraction (SPE) on C_{18} disposable extraction cartridges (DECs). All the sample handling operations were effected by a robotic auto-sampler. The DEC was first conditioned with methanol and phosphate buffer pH 7.4. After loading 1.0 ml of plasma sample onto the DEC, the latter was washed with the same buffer. The elution step was performed with methanol (0.25 ml) and the eluate was then diluted by adding 0.75 ml volume of phosphate buffer pH 6.4. A 20-μl volume of the resultant solution was injected onto an octadecyl silica column preceded by a short guard column. The HPLC mobile phase was methanol–phosphate buffer pH 6.4 (25:75, v/v). Sulfamethazine and N_4-acetylsulfamethazine were determined photometrically at 262 nm. Under these conditions, linear calibration curves ranging from 2 to 250 μg ml^{-1} have been obtained for both compounds. Drug recoveries were higher than 90% and typical relative standard deviation values were 0.7% (within-day) and 2.0% (between-day) at a plasma concentration of 50 μg ml^{-1}.

INTRODUCTION

Sulfamethazine [sulfadimidine or sulfamezathine or 4-amino-N-(4,6-dimethyl-2-pyrimidine) benzenesulfonamide] (Fig. 1) is one of the most commonly used sulfonamides in veterinary medicine for therapeutic and prophylactic purposes in diseases of food-producing animals. The metabolism of sulfamethazine (SMZ) involves acetyla-

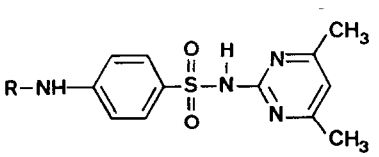

Fig. 1. Structures of (1) sulfamethazine (SMZ) and (2) N_4-acetyl sulfamethazine

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tion of the *p*-amino group (N_4-SMZ) (Fig. 1) [1,2] and hydroxylation of one of the methyl groups on the pyrimidine ring (6-hydroxymethyl-SMZ) or of the pyrimidine ring itself (5-hydroxy-SMZ) followed by glucuronidation [3–5]. The plasma protein binding of sulfamethazine and its metabolites depends on their plasma concentration range. At high concentrations (>50 μg ml^{-1}), the binding percentage for SMZ is smaller (*ca.* 51%) that at low concentrations (about 79%) [6].

Photometric methods have been widely used for the determination of sulfonamides in plasma which were based on diazotation of the primary arylamino group and photometric determination of the azodye formed through coupling with a suitable reagent [N-(1-naphtyl ethylene diamine) dihydrochloride] [7]. Various procedures have also been published to determine different sulfonamides in biological fluids by HPLC [2,4–6,8–15]. Among these methods, protein precipitation with a strong acid is the most frequently applied technique for sample preparation [2,4–6,11,12]. In some cases, off-line liquid–liquid extractions of the analytes with an organic solvent [13] after basification [14] have been proposed. Two other methods involve liquid–solid extraction as sample preparation procedure using either a gel chromatography on a Sephadex G25 glass column [10] or on an Extrelut 1 column [15]. The possibility of enhancing the selectivity and sensitivity of detection by use of fluorometry [14] or amperometry [15] instead of UV spectrophotometry has been sometimes exploited.

In this paper, a fully automated technique for the determination of sulfamethazine and its N_4-acetyl metabolite in ovine plasma after single oral administration of a sulfamethazine bolus [16,17] is described. The sample cleanup is performed by solid-phase extraction (SPE) on disposable extraction cartridges (DECs). All the operations are effected by an autosampler equipped with a robotic arm to which is attached a needle dispensing the different liquids [17,18]. The dependence of the drug recovery on the plasma dispensing flow-rate and the efficiency of two mixing modes applied to homogenize the final extract have been especially studied. The method developed has been validated and analytical data are presented.

EXPERIMENTAL

Apparatus

Solid-phase extraction was performed on DECs and the plasma samples were treated in the same way as in a manual procedure by an ASPEC (Automated Sample Preparation with Extraction Cartridges) system from Gilson (Villiers-le-Bel, France). This fully automated system consists of three components: a sample processor, a Model 401 dilutor/pipettor and a set of racks and accessories to handle DECs. The mobile DEC holder located on the SPE rack automatically places each DEC either above a drain cuvette or collection tube, depending on the extraction step being performed. The collected fraction is then introduced into the 20-μl loop of an electrically actuated injection valve for on-line HPLC analysis. The dispensing flow-rates of liquids used for the sample handling can be varied from 0.18 to 96.0 ml min^{-1}. The ASPEC system uses positive air pressure to push the different liquids through the DEC with a special cap ensuring an air-tight fit while solvent, sample or air is dispensed through the needle. The air pressurizing volumes can be changed, the passage of liquid through the DEC being accelerated by the use of larger air volumes.

The liquid chromatographic system consisted of a Gilson Model 305 pump, a Gilson Model 805 manometric module and a Spectra 200 UV-Vis programmable wavelength detector from Spectra-Physics (San Jose, CA, USA).

The separation was performed on a LiChroCART analytical column (125 × 4 mm I.D.) preceded by a short LiChroCART guard column (4 × 4 mm I.D.) from E. Merck (Darmstadt, Germany). They were thermostatted at 35.0 ± 0.1°C in a Model VC 20B water-bath (Julabo Labortechnik, Seelbach, Germany).

The HPLC instrumentation and the ASPEC system were interfaced to an IBM-AT microcomputer (CPU type: 80386) equipped with 4 Mbyte RAM, a 105 Mbyte hard disk and a Hewlett-Packard Deskjet 500 printer. The con-

trol of the instrumentation was effected through a resident Gilson GME-715 version 1.1 software.

Chemicals and reagents

Sulfamethazine was purchased from Sigma (Saint-Louis, MO, USA). Synthesis of N_4-acetyl sulfamethazine was based on the procedure described by Bary *et al.* [13]. The purity of the product was checked by elementary analysis, IR spectroscopy and by HPLC with UV detection.

Sodium hydroxide and potassium dihydrogen phosphate were of analytical reagent grade from Merck. HPLC-grade water was produced in a Milli-Q water purification system (Millipore, Bedford, MA, USA) and was used throughout this work. Methanol from Janssen (Geel, Belgium) was also of HPLC grade.

The DECs used were Bond Elut cartridges from Analytichem (Harbor City, CA, USA), filled with 100 mg of octadecyl phase (particle size: 40 μm). The LiChroCART guard and analytical columns were both prepacked with LiChrospher 100 RP18, 5 μm (Merck).

Chromatographic technique

The mobile phase consisted of methanol–0.05 *M* phosphate buffer pH 6.4 (25:75, v/v) [17] and was degassed by using an ultrasonic bath for 15 min. The flow-rate was 0.9 ml min^{-1}. Prior to use, the phosphate buffer (pH 6.4) was passed through a 0.45-μm membrane filter from Schleicher & Schuell (Dassel, Germany). The analytes were simultaneously monitored photometrically at 262 [17] and 292 nm.

Standard solutions

A stock solution containing 5.0 mg ml^{-1} of SMZ and its N_4-acetyl metabolite in water was prepared. It was further diluted with water to concentrations of 1.0 mg ml^{-1} and 0.2 mg ml^{-1}. All these solutions were stored in a refrigerator at 4°C. Working standard solutions were prepared in the range 1–375 μg ml^{-1} by diluting aliquots of the stock solutions with the HPLC mobile phase. They were used to perform direct injections in order to evaluate the absolute recoveries of the analytes at different concentrations.

Spiked plasma samples

Aliquots of the three stock solutions were employed to spike 2.0 ml blank plasma samples in the concentration range studied (1–375 μg ml^{-1}). After vortex-mixing, the spiked plasma samples were left to stand for 30 min before solid-phase extraction was carried out.

Automatic sample preparation

A 2.0-ml volume of plasma was transferred manually into a vial which was then placed on the appropriate rack of the ASPEC system. The needle of the sample processor and the external tubing of the injection valve were washed with 2.0 ml of phosphate buffer (pH 7.4) before the starting of the first cycle. Between each step, the needle was rinsed with a 1.0-ml volume of buffer and a 10-mm air gap was generated inside the transfer tubing before pipetting the next liquid, in order to avoid cross-contamination.

The automatic sample preparation, an outline of which is given in Table I, was performed as follows (total run time: 15.6 min):

- *Conditioning of the DEC* (flow-rate: 12.0 ml/min; air volume: 0.3 ml): The DEC (C_{18}, 100 mg), placed in the DEC holder positioned above the drain cuvette, was first conditioned with 2.0 ml of methanol and 2.0 ml of phosphate buffer (pH 7.4).
- *Loading of the plasma sample* (flow-rate: 0.75 ml/min; air volume: 1.5 ml): A 1.0-ml volume of plasma was applied onto the DEC.
- *Washing* (flow-rate: 1.5 ml/min; air volume:

TABLE I

AUTOMATIC SAMPLE PREPARATION PROCEDURE[a]

Step	Liquid	Volume (ml)
Conditioning	Methanol buffer pH 7.4	2.00
		2.00
Sample loading	Plasma	1.00
Washing	Buffer pH 7.4	2.00
Elution	Methanol	0.25
Buffer addition and mixing	Buffer pH 6.4	0.75
Injection	Plasmatic extract	0.02

[a] DEC: Bond Elut C_{18} (100 mg, 1 ml-capacity)

1.0 ml): 2.0 ml of phosphate buffer pH 7.4 were passed through the DEC.
- *Elution of the analytes* (flow-rate: 1.5 ml/min; air volume: 1.1 ml): The DEC holder was moved by the needle of the robotic arm above the part of the SPE rack containing collection tubes. A 0.25-ml volume of methanol was dispensed on the DEC and the eluate was collected into the corresponding tube.
- *Addition of buffer* (flow-rate: 1.5 ml/min; air volume: 0.6 ml): A 0.75-ml volume of phosphate buffer pH 6.4 was passed through the DEC.
- *Mixing*: After displacement of the DEC holder to its initial position, the resultant solution was homogenized by aspirating and dispensing it three times in the collection tube (pumping mode)
- *Injection*: The final extract was introduced into the loop filler port of the injection valve and then injected onto the HPLC column by valve switching (injection loop: 0.02 ml).

The liquid chromatographic separation of the prepared sample was performed during the preparation of the next sample (concurrent mode).

RESULTS AND DISCUSSION

Automated sample preparation procedure

The fully automated SPE procedure using the ASPEC system, described in this paper, has been developed according to the general principles presented earlier [18–21]. As used previously in a similar method for the bioanalysis of indomethacin [18], a disposable extraction cartridge packed with octadecylsilica (100 mg) was selected as well as a phosphate buffer pH 7.4 for the conditioning and washing steps. In order to obtain a final extract with the same eluting strength as the HPLC mobile phase, 0.25 ml of methanol and subsequently 0.75 ml of phosphate buffer pH 6.4 have been used. Phosphate buffer pH 6.4 has been preferred for the dilution of the methanolic eluate (see Table I), because it has been observed that differences in pH between the final extract and the HPLC mobile phase could give rise to disturbing system peaks [20]. Under these experimental conditions, higher recoveries and very clear chromatograms devoid of interfering peaks (*cf.* Fig. 4) from plasma components have been obtained. In addition, due to the high molar absorptivity of the analytes at the measuring wavelength and their use in a relatively high plasma concentration range, no further optimization of the elution step, as described earlier [21], was found to be necessary. Consequently, these conditions have been applied as such to the automatic determination of sulfamethazine and its acetyl metabolite in ovine plasma.

Dispensing flow-rate on the DEC

The dispensing flow-rates of the different liquids used in the SPE procedure are generally not critical, except for the delivery of the plasma sample [18]. For drugs strongly bound to plasma proteins, the minimum dispensing flow-rate available (0.18 ml min^{-1}) has to be selected for plasma application in order to obtain sufficient recoveries (>90%) [18]. Indeed, at high dispensing flow-rates, the absolute recovery decreases drastically with increasing dispensing flow-rate due to the fact that the residence time of the plasma sample in the DEC is reduced to such an extent that a part of the drug is displaced from its binding to proteins and can be distributed to the DEC sorbent.

As could be expected with sulfamethazine, which has a degree of binding to plasma of *ca.* 50% at the working plasmatic concentration (50 $\mu g\ ml^{-1}$), Fig. 2 shows that a much less pronounced decrease in recovery is obtained with

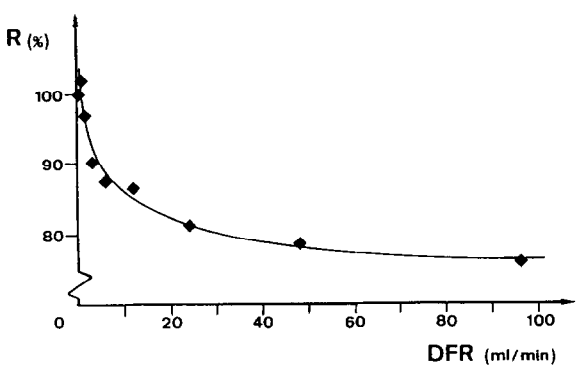

Fig. 2. Influence of the dispensing flow rate of plasma on the recovery of SMZ DEC: Bond Elut C_{18} (100 mg), SMZ plasma concentrations: 50 $\mu g\ ml^{-1}$. R: absolute recovery of SMZ; DFR: dispensing flow-rate of the plasma sample.

increasing dispensing flow-rate. In this case, a dispensing flow-rate of 0.75 ml min^{-1} could be used while keeping a high recovery. The total cycle time of the sample preparation is then reduced, which results in an increased sample throughput.

Mixing mode

After elution of the drug from the DEC by methanol and addition of HPLC buffer (*cf.* Table I), the final extract is not homogeneous as illustrated in Fig. 3. A mixing step is thus required before injection of the extract into the HPLC system. The two available mixing modes are pumping or air bubbling. The influence of the repetition of these respective mixing modes on the analyte recovery has been studied and results are presented in Fig. 3. Two or three air volumes (1.0 ml) or three pumping steps are necessary to obtain an optimal mixing process. The efficiency of the two mixing modes is thus approximately equivalent but the pumping mode has finally been preferred to bubbling in order to avoid losses of liquid by spattering out of the collection tube.

Validation of the method

Typical chromatograms obtained from a drug-free plasma sample and from a plasma spiked with 25 $\mu g\ ml^{-1}$ of each compound worked up according to the sample handling procedure are shown in Fig. 4. The retention times of sulfamethazine and N_4-acetyl sulfamethazine were 4.6 and 6.7 min, respectively.

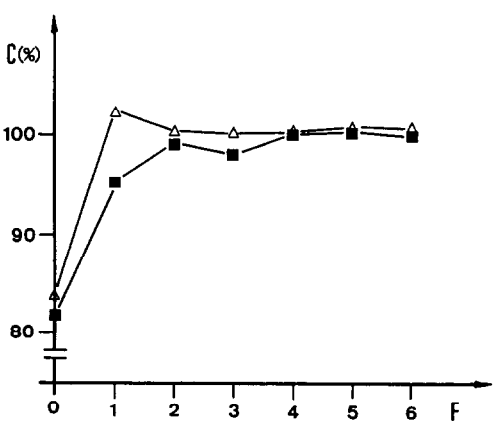

Fig. 3. Efficiency of both mixing modes. DEC: Bond Elut C_{18} (100 mg); Dispensing flow-rate of the liquids: 1.5 ml min^{-1}; Other conditions as given in Experimental. (△) pumping, (■) bubbling. C: relative concentration of SMZ; F: number of repetitions.

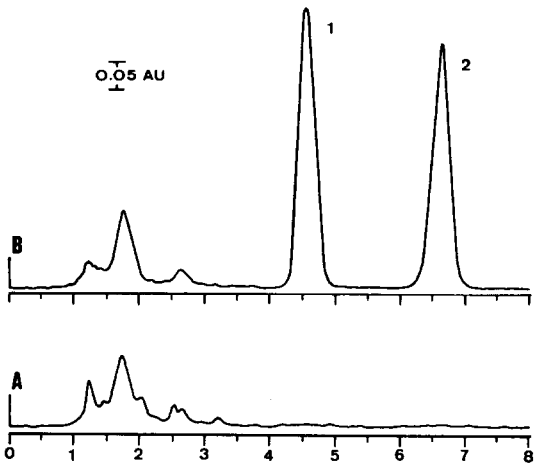

Fig. 4. Typical chromatograms of extracts from (A) blank plasma, (B) spiked plasma with 25 $\mu g\ ml^{-1}$ obtained by using SPE on DECs coupled to HPLC. DEC: Bond Elut C_{18} (100 mg; 1-ml capacity); Dispensing flow-rate for plasma sample: 0.75 ml min^{-1}; Other chromatographic conditions as described in Experimental. Peaks: (1) sulfamethazine (SMZ). (2) N_4-acetylsulfamethazine (N_4-SMZ).

Absolute recovery. As can be seen in Table II, the absolute recoveries of the drugs studied have been determined at nine different concentrations ranging from 1 to 375 $\mu g\ ml^{-1}$. The mean absolute recovery of sulfamethazine and N_4-acetyl sulfamethazine are 97% and 93%, respec-

TABLE II

ABSOLUTE RECOVERY OF THE DRUGS[a]

Concentration (μg/ml)	SMZ (%)	N_4-SMZ (%)
1	102	102
5	92	92
10	96	99
25	95	90
50	100	91
75	96	93
125	99	89
250	98	92
375	96	91
Mean ± S.D.	97 ± 3	93 ± 4

[a] Each value represents the mean of two determinations.

tively. The absolute recoveries were calculated by comparing the peak areas obtained from freshly prepared spiked plasma sample extracts to those obtained by the analysis of aqueous standard solutions of the same concentration injected directly into the HPLC system by use of the same autosampler [22].

Linearity. The calibration curves were constructed in the range 2–250 μg ml^{-1} ($n = 7$, AUFS = 0.05). Linear regression analysis made by plotting the area (y) (arbitrary unit) *versus* the concentration (x) in μg ml^{-1} gives the following equations:

SMZ: $y = 1557.1(\pm 2.54)x - 25.8(\pm 139.8)$

$r^2 = 0.9999$

N_4-SMZ: $y = 1532.0(\pm 12.9)x + 657.1(\pm 708.3)$

$r^2 = 0.9997$

The very good linearity of the calibration curves is demonstrated by the determination coefficients (r^2) obtained for the regression lines.

Reproducibility. As shown in Table III, the precision of the bioanalytical method was estimated by measuring the within-day and between-day reproducibilities of the analytes at three concentration levels which are frequently encountered in the present bioavailability studies. The very good R.S.D. values are to be related to the use of a fully automated method and to the fairly high plasma concentration range studied.

Accuracy. Accuracy was determined by analyzing spiked plasma samples at three different concentrations and comparing the experimentally measured values to the nominal concentrations. The bias of the method expressed as the relative error of measurement (R.E.M.) is given in Table III. Mean values are very close to the theoretical concentrations, showing method accuracy ranging from 0.9 to 4.2%.

Detectability. As proposed by Miller and Miller [23], the limits of detection (LOD) and quantitation (LOQ) were calculated from regression line in the following way:

$$LOD = Y_B + 3s_B \quad LOQ = Y_B + 10s_B$$

where Y_B was the blank signal (intercept) and s_B the standard deviation of the blank ($S_{y/x}$) obtained with calibration curve ranging from 1 to 250 μg ml^{-1}. The LOD of sulfamethazine is equal to 1.1 μg ml^{-1} and its LOQ to 3.7 μg ml^{-1}. The LOD and LOQ of N_4-acetyl sulfamethazine are respectively equal to 1.5 μg ml^{-1} and 5.0 μg ml^{-1}.

The limits of detection could certainly have been lowered by a proper reduction of the volumes of methanol and phosphate buffer used in the elution step [21] or simply by increasing the volume of the injection loop used (>0.02 ml), but such an improvement in sensitivity was not needed in this case due to the fact that the sulfamethazine therapeutic level in ovine plasma is higher than 25 μg ml^{-1} [24].

TABLE III

PRECISION AND ACCURACY OF THE AUTOMATED METHOD

Plasma level (μg ml^{-1})	n	Sulfamethazine		N_4-Sulfamethazine	
		R.S.D. (%)	R.E.M. (%)	R.S.D. (%)	R.E.M. (%)
Within-day					
25	7	1.2	2.3	1.3	4.2
50	5	0.7	1.6	1.0	0.9
125	5	1.7	1.7	1.5	1.6
Between-day					
25	7	2.0	2.5	2.9	3.8
50	6	2.0	2.4	3.4	2.8
125	5	1.6	1.4	2.1	1.8

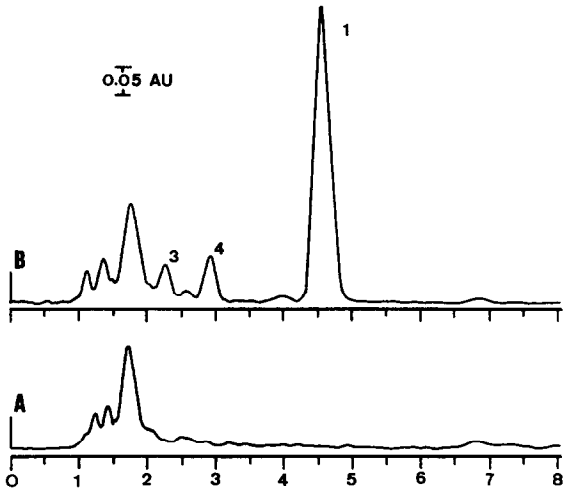

Fig. 5. Typical HPLC chromatograms of ovine plasma: (A) before dosing (control); (B) 48 h after oral administration of sulfamethazine bolus. The calculated concentration of sulfamethazine was 28 μg ml^{-1}; SPE and chromatographic conditions were as indicated in the text. Peaks: (1) sulfamethazine (SMZ), (3 + 4) unknown peaks (possibly corresponding to two hydroxy metabolites of sulfamethazine).

Selectivity. Fig. 5 shows chromatograms obtained by analyzing plasma samples collected from a particular lamb before and after receiving oral administration of a sulfamethazine bolus (48 h after dosing). In Fig. 5(B) two small unknown peaks appear at retention times lower than that of sulfamethazine, possibly corresponding to the hydroxy metabolites (5-hydroxy sulfamethazine and 6-methylhydroxy sulfamethazine) [6]. No interferences from endogenous compounds or potential metabolites have been observed in these samples and those from other lambs participating in the study. In addition, during routine analysis, the analytes were monitored at two different wavelengths (262/292 nm) and the ratio of the absorbances was registered continuously in order to test peak homogeneity.

Application of the automated method

An example of the time course of sulfamethazine concentration in plasma from healthy lambs ($n = 4$) after intravenous administration of 100 mg kg^{-1} is given in Fig. 6. The mean elimination half life ($t_{1/2}$) and the apparent volume of distribution (V_D) of sulfamethazine

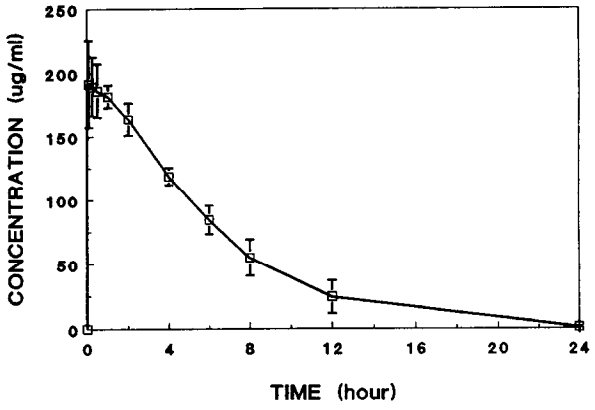

Fig. 6. Mean sulfamethazine concentration-time profile following an intravenous dose of 100 mg kg^{-1} in four healthy lambs.

were 4.04 h and 0.526 l kg^{-1}, respectively. The mean area under the plasma concentration time curve (AUC) determined at infinity was equal to 1284 ± 203 μg h ml^{-1}. Fig. 6 also demonstrates that the method is sensitive enough for the quantitative determination of sulfamethazine in biological samples. Detailed pharmacokinetic analysis after oral administration of a sulfamethazine bolus will be published later. In the framework of these bioavailability studies in lambs, the automated method has been applied successfully to more than five hundred plasma samples and has proved to be rugged.

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REFERENCES

1 R.F. Bevill, L. Dittert and D.W.A. Bourne, *J. Pharm. Sci.*, 66 (1977) 619.
2 J.F.M. Nouws, T.B. Vree, M. Baakman and M. Tijhuis, *Vet. Q.*, 5 (1983) 41.
3 P. Nielsen, *Biochem. J.*, 136 (1973) 1039.
4 T.B. Vree, M. Tijhuis, J.F.M. Nouws and Y.A. Hekster, *Pharm. Weekbl.*, 6 (1984) 80.
5 J.F.M. Nouws, T.B. Vree, M.J. Breukink, M. Baakman, F. Driessens and A. Smulders, *Vet. Q.*, 7 (1985) 177.

6 J.F.M. Nouws, T.B. Vree, M. Baakman, F. Driessens, H.J. Breukink and D. Mevius, *Am. J. Vet. Res.*, 47 (1986) 642.
7 A.C. Bratton and E.K. Marshall, *J. Biol. Chem.*, 128 (1939) 537.
8 T.B. Vree, Y.A. Hekster, A.M. Baars, J.F. Damsma and E. van der Kleijn, *J. Chromatogr.*, 146 (1978) 103.
9 K. Harzer, *J. Chromatogr.*, 155 (1978) 399.
10 B. Salvesen and P. Nordbo, *Medd. Norsk. Farm. Selsk.*, 41 (1979) 15.
11 R.L. Suber and G.T. Edds, *J. Liq. Chromatogr.*, 3 (1980) 237.
12 R.T.M. Van Der Hoeven, *Pharm. Weekbl.*, 117 (1982) 845.
13 J.D. Baty, R.M. Lindsay and S. Sharp, *J. Chromatogr.*, 353 (1986) 329.
14 M. Petz, *J. Chromatogr.*, 423 (1987) 217.
15 D. Von Baer, A. Momberg, M.E. Carrera, R. Arriagada and M.R. Smyth, *J. Pharm. Biomed. Anal.*, 9 (1991) 925.
16 B. Evrard, J.P. Dechesne, X. Fargetton, N. Zygraich and L. Delattre, *Paper presented at the 5th International Conference on Pharmaceutical Technology, Paris, France, 30 May–1 June*, Proc. Vol. I (1989) 294.
17 B. Evrard, J.P. Dechesne, X. Fargetton and L. Delattre, *J. Pharm. Belg.*, 45 (1990) 72.
18 Ph. Hubert and J. Crommen, *J. Liq. Chromatogr.*, 13 (1990) 3891.
19 Ph. Hubert, P. Chiap and J. Crommen, *J. Pharm. Biomed. Anal.*, 9 (1991) 877.
20 Ph. Hubert, M. Renson and J. Crommen, *J. Pharm. Biomed. Anal.*, 7 (1989) 1819.
21 Ph. Hubert, P. Chiap and J. Crommen, *J. Pharm. Biomed. Anal.*, 9 (1991) 883.
22 A.R. Buick, M.V. Doig, S.C. Jeal, G.S. Land and R.D. McDowall, *J. Pharm. Biomed. Anal.*, 8 (1990) 629.
23 J.C. Miller and J.N. Miller, in R.A. Chalmers and M. Masson (Editors), *Statistics for Analytical Chemistry*, Ellis Horwood, Chichester, 1984, p. 96.
24 M. Nawaz and F.H. Khan, *J. Vet. Pharmacol. Ther.*, 2 (1979) 129.