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Semi-automated solid-phase extraction procedure for the high-performance liquid chromatographic determination of alinastine in biological fluids

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Abstract

A solid-phase extraction (SPE) method for sample clean-up followed by a reversed-phase HPLC procedure for the assay of alinastina (pINN) in biological fluids is reported. The effects of the sample pH, composition of the washing and elution solvents and the nature of the SPE cartridge on recovery were evaluated. The selectivity of SPE was examined using spiked rat urine and plasma samples and the CH and PH cartridges gave rise to the cleanest extracts. The recoveries obtained in spiked rat urine and plasma samples were 91.2±2.7 and 99.9±2.8%, respectively. The proposed SPE method coupled off-line with a reserved-phase HPLC system with fluorimetric detection was applied to the quantitation of alinastine in real rat urine samples. The analytical method was also applied and validated for the determination of alinastine in dog plasma. The recovery from spiked dog plasma samples using the PH cartridge was around 65%. The within-day and between-day precisions were 7 and 12%, respectively. The detection and quantitation limits in dog plasma were 0.024 and 0.078 μg/ml, respectively. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Alinastine

1. Introduction

The purpose of this article is to describe a semi-automated method developed for the determination of alinastine (pINN) (Fig. 1) in biological fluids. Alinastine is a new antihistamine drug not yet marketed and under biopharmalogical study. No analytical methodology for its assay in biological fluids has been described to date. So, this analytical method is necessary to develop pharmacokinetic studies of this compound in laboratory animals and humans. The proposed method involves sample handling by SPE and off-line injection of the extracts

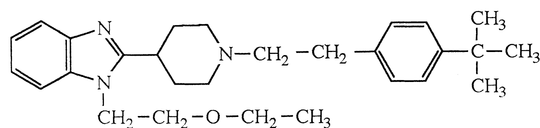

Fig. 1. Structure of alinastine, 2-(1-(2-(4-(1,1-dimethylethyl)phenyl)ethyl)piperidin-4-yl)-1-(2-ethoxyethyl)-1*H*-benzimidazole.

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into a HPLC system with fluorescence detection of the analyte.

Biological matrices need an elaborate and labour-intensive sample clean-up procedure to remove unwanted substances [1–4]. Moreover, sample preparation is a critical step for the precision and accuracy of the analytical procedure. Solid-phase extraction (SPE), as a convenient and powerful tool for sample clean-up, is widely used in the analysis of drugs in biological samples [5–14]. In recent years, SPE has not only been used for a single drug or a series of analogous compounds [15–17], but also for the screening of a wide range of drugs [18,19]. The advantages of SPE over traditional liquid–liquid extraction (LLE) have been well documented in many publications (e.g., Ref. [1]). One of its major advantages is that the extraction procedure can be automated. Several semi- or fully automated SPE methods have been reported [20–23].

In this article the extraction efficiency of the SPE for alinastine has been investigated in aqueous standard samples with several solid-phase extraction cartridges (C_{18}, C_8, C_2, CH and PH), variable pH values (2, 4, 6, 7, 8 and 10) and different methanol–water mixtures as eluent and washing liquids (50:50, 60:40, 70:30, 80:20, 90:10 and 100:0). Using the optimum SPE conditions the selectivity of the different cartridges in spiked rat plasma and urine has been studied. Finally, the method for determining alinastine in dog plasma has been validated.

2. Experimental

2.1. Reagents

Alinastine and the internal standard (F-91403-RR) were supplied by FAES, S.A. (Bilbao, Spain). Methanol and acetonitrile (Romil, Harvehill, UK) were of HPLC grade. The water used in all the experiments was purified on a Milli-Q system from Millipore (Bedford, MA, USA). Sodium acetate, acetic acid, phosphoric acid, sodium hydrogen and dihydrogen phosphates, boric acid, sodium borate, sodium hydroxide and triethylamine were of analytical quality from Merck (Darmstadt, Germany). Aqueous buffers were prepared in order to maintain the pH of the aqueous standard and the spiked urine and plasma samples. The buffers were: H_3PO_4–NaH_2PO_4 (pH 2), HAc–NaAc (pH 4–5), NaH_2PO_4–Na_2HPO_4 (pH 6–8) and $NaHCO_3$–Na_2CO_3 (pH 10). Stock standard solutions of alinastine and F-91403-RR were prepared in methanol at a concentration of 1000 μg/ml and were stored at 4°C in the dark. These stock solutions were stable under these storage conditions for almost 6 months. Working solutions were prepared daily by dilution from stock solutions.

2.2. Instrumentation

The SPE cartridges used were Bond-Elut (Scharlau, Barcelona, Spain) octadecyl (C_{18}), octyl (C_8), ethyl (C_2), cyclohexyl (CH) and phenyl (PH) silica-bonded phases (100 mg). The extraction was performed with ASPEC XL equipment from Gilson (Villiers le Bel, France).

HPLC was used for the determination of the recoveries. The chromatographic system consisted of a LKB (Barcelona, Spain) 2150 pump, a Rheodyne (Cotati, CA, USA) 7125 sample injector with a fixed loop of 50 μl, a Perkin-Elmer (Norwalk, CT, USA) LC101 column oven and a Shimadzu (Kyoto, Japan) RF-540 spectrofluorimeter equipped with an HPLC cell (12 μl). A reversed-phase Hewlett-Packard (Palo Alto, CA, USA) Asahipak ODP-50 column (125 mm×4 mm, 5 μm I.D.) was used. The excitation and emission wavelengths were 265 and 340 nm, respectively. The column temperature was 40°C. The mobile phase was acetonitrile–0.01 *M* triethylamine (pH 2.3 adjusted with 15 *M* H_3PO_4) (20:80, v/v) delivered at a flow-rate of 1.2 ml/min.

2.3. Extraction procedure

2.3.1. SPE studies in aqueous standard samples

Buffered aqueous samples of alinastine at a concentration of 0.5 μg/ml were prepared by dilution from the stock solutions using the appropriate aqueous buffer. The extraction was performed by the ASPEC system in sequential mode in the following way. The SPE cartridge was activated with 1 ml methanol and 1 ml aqueous buffer. Then, 1 ml of sample was loaded and the compound of interest was eluted with 1 ml of a suitable elution solvent. For the study of the optimal pH, the elution solvent was

Table 1
Recoveries of alinastine from aqueous standard samples [% ±relative standard deviation (RSD) of $n=3$ determinations] using the different cartridges as a function of the sample pH

	pH						
	2.0	4.0	5.0	6.0	7.0	8.0	10.0
C_{18}	12.8±1.4	51.28±0.31	58.2±1.0	64.9±1.5	53.4±2.2	39.09±0.16	15.41±0.26
C_8	18.1±7.5	11.56±0.43	69.8±1.7	63.7±1.3	52.14±0.77	35.7±1.8	2.69±0.32
C_2	15.2±1.0	13.8±2.4	56.90±0.36	62.6±2.3	49.48±0.65	28.6±1.5	11.27±0.38
CH	9.39±0.26	35.9±1.7	70.4±2.5	56.96±0.42	48.9±1.6	32.5±2.4	
PH	4.260±0.013	42.98±0.38	59.2±2.7	58.51±0.57	35.0±1.2	17.48±0.59	

methanol and for the elution study (recoveries evaluated as a function of the composition of the elution solvent) methanol–aqueous buffer mixtures were used.

2.3.2. SPE studies in spiked rat urine and plasma samples

Blank urine and plasma samples were spiked with 0.5 μg alinastine per ml of biological fluid and buffered with the appropriate buffer. The SPE procedure was similar to the case of the aqueous standard samples, but the cartridge was washed with 1 ml of a convenient washing solvent prior to the elution with 1 ml of an adequate elution solvent.

The extracts from all studies were evaporated to dryness in a waterbath at 40°C with the aid of a Visiprep Vacuum Manifold (Supelco, Bellefonte, USA) and reconstituted with 200 μl of mobile phase containing 10 μg/ml of internal standard. Aliquots (50 μl) of this solution were injected into the chromatographic system. Recoveries were calculated against the initial amount of alinastine spiked in the sample (aqueous, urine or plasma) using linear calibration curves based on pure solutions of alinastine and internal standard.

3. Results and discussion

The recoveries obtained in the study of the effect on recovery of sample pH using different cartridges (C_{18} C_8, C_2, CH and PH) are presented in Table 1 and show that the substance studied is more retained extension with all the cartridges at pH between 5 and 6.

The recoveries attained with each cartridge were then evaluated as a function of the composition of the eluent mixture (methanol–water), fixing the sample pH at 6.0. Results are shown in Table 2, which gives information on the best composition of the washing solvent (that with the highest percentage of methanol without elution of the compound) and the best elution solvent (the lowest percentage of methanol that gives maximum recovery of the compound). The correct selection of the washing and elution solvents will provide the cleanest samples in the SPE process and therefore the best selectivity in the extraction. The optimum conditions in the aqueous standard study for alinastine were 1 ml methanol–water (70:30, v/v) as the washing solvent and 1 ml methanol as the eluent solvent for all types of cartridges.

Table 2
Recoveries of alinastine from aqueous standard samples (% ±RSD of $n=3$ determinations) using the different cartridges at pH 6.0 as a function of the composition of the elution mixture (methanol–water)

	Methanol–water					
	50:50	60:40	70:30	80:20	90:10	100:0
C_{18}	<0.4	<0.4	<0.4	<0.4	36.9±2.0	64.1±3.0
C_8	<0.4	<0.4	<0.4	<0.4	39.11±0.52	61.6±1.0
C_2	<0.4	<0.4	3.00±0.67	36.9±1.6	48.7±1.7	62.0±1.9
CH	<0.4	<0.4	<0.4	3.52±0.72	37.43±0.96	58.8±2.7
PH	<0.4	<0.4	<0.4	6.6±4.9	39.27±0.29	58.5±1.2

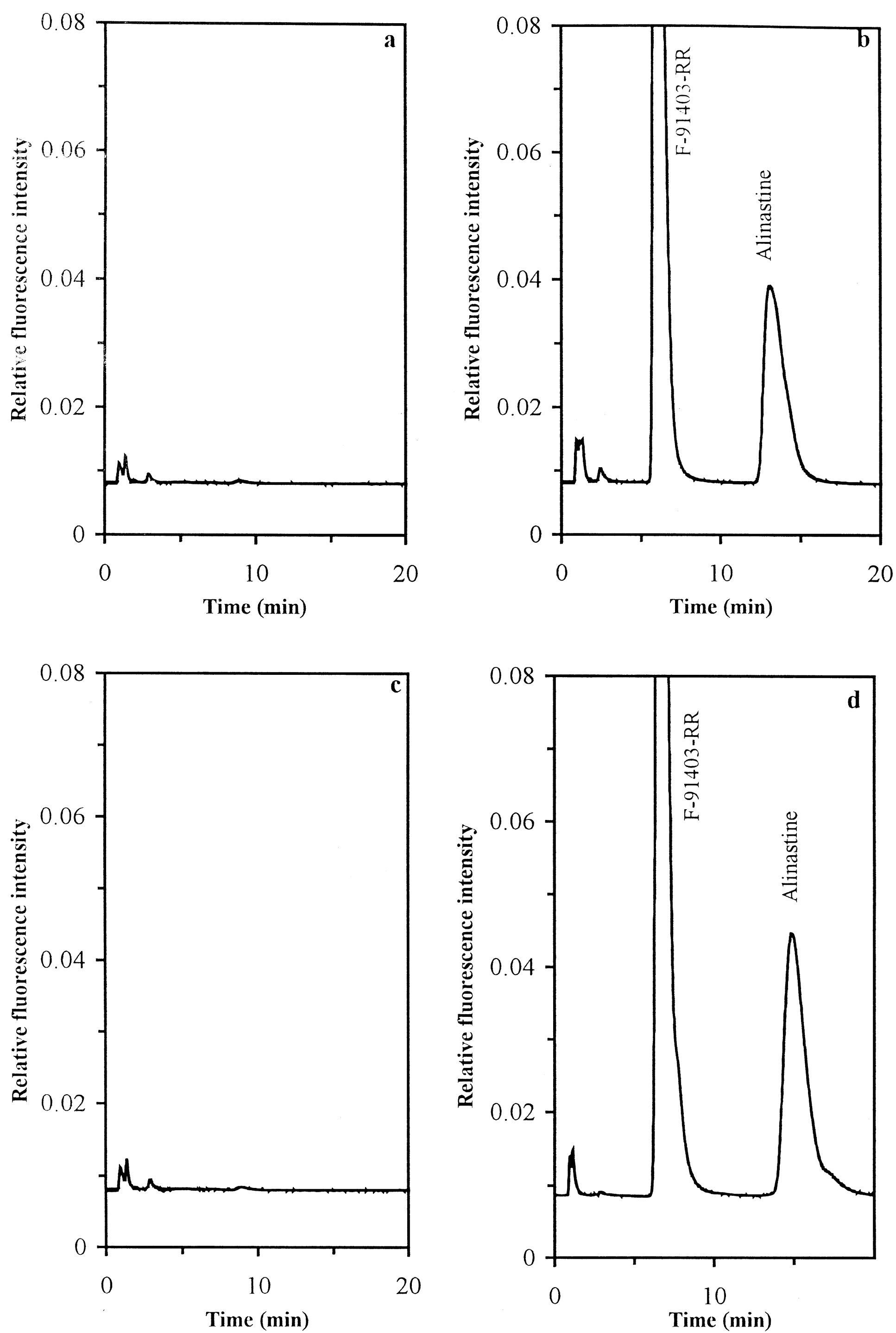

Fig. 2. Typical chromatograms obtained for: (a) blank rat urine, (b) urine sample spiked with 0.5 μg alinastine per ml, (c) blank rat plasma and (d) plasma sample spiked with 0.5 μg alinastine per ml.

In order to select the most appropriate sorbent in relation to selectivity, rat urine and plasma samples spiked with 0.5 μg alinastine per ml, buffered at optimum pH (5 or 6 depending on the nature of the cartridge), were applied on each type of SPE cartridge. With all cartridges the obtained selectivity was suitable, but the cleanest chromatograms and the best recoveries for the spiked urine and plasma samples were obtained using cyclohexyl silica (CH) for urine samples and phenyl silica (PH) for plasma samples. The recoveries obtained were 91.2±2.7% for urine and 99.9±2.8% for plasma.

Thus, the optimum conditions finally proposed for the SPE of alinastine in biological fluids were:

- *Cartridge conditioning* (flow-rate, 3.0 ml/min; air volume, 0.2 ml): the cartridge (CH for urine samples and PH for plasma samples) was first conditioned with 1.0 ml methanol and then with 1.0 ml phosphate buffer (pH 6.0 for plasma samples and pH 5.0 for urine samples).
- *Loading with sample* (flow-rate, 1.50 ml/min; air volume, 0.2 ml): 1 ml sample buffered with phosphate buffer (pH 6.0 for plasma samples and pH 5.0 for urine samples) was dispensed on the cartridge.
- *Washing* (flow-rate, 3.0 ml/min; air volume, 3 ml) with 1 ml methanol–water (70:30, v/v). The column was dried by passing 3 ml of air through it.
- *Elution* (flow-rate, 0.2 ml/min; air volume, 0.2 ml) with 1 ml methanol.

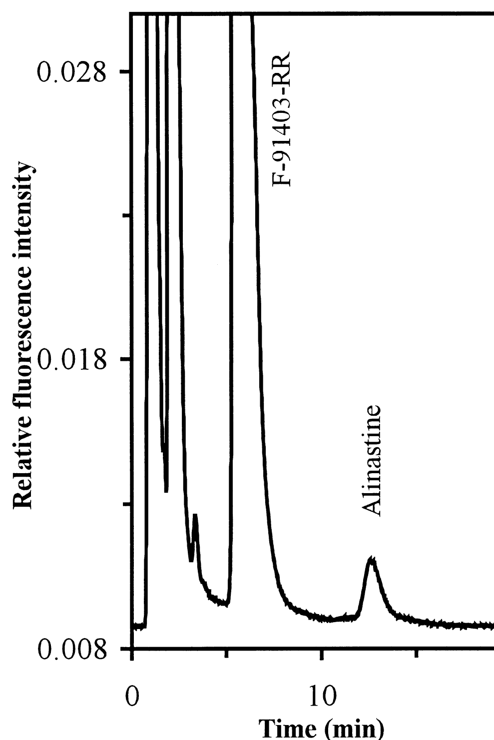

Fig. 3. Chromatogram of a real rat urine sample collected between 8 and 24 h of oral administration of 57.1 mg alinastine/kg.

Typical chromatograms for blank rat urine and plasma extracts and spiked rat urine and plasma extracts are given in Fig. 2. Fig. 3 shows the chromatogram corresponding to a sample of rat urine collected between 8 and 24 h of oral administration of 57.1 mg alinastine per kilogram. The amount of alinastine found in this sample was 0.11 μg/ml

The SPE method was also applied and validated for dog plasma samples. The mean recovery for alinastine (at four different concentrations ranging from 0.1 to 5 μg/ml) in spiked dog plasma was around 65%. The precision of the bioanalytical method, expressed by the CV, was estimated by measuring the within-day and between-day reproducibilities of the analysis for plasma samples spiked at five concentrations ranging from 0.03 to 5 μg/ml. The within-day reproducibility ranged from 3.2 to 6.8%, whereas the between-day reproducibility ranged from 5.6 to 11.7% (Table 3).

Blank dog plasma was spiked at different concentrations and carried through the extraction procedure, giving a linear calibration curve, at least in the concentration range 0.03–5 μg/ml. The limit of detection (LOD), calculated as the analyte concentration that produced a chromatographic peak with a height three times the baseline noise (3 S/N), was 0.024 μg/ml, and the quantitation limit, calculated as the analyte concentration that produced a chromatographic peak with a height 10 times the baseline noise (10 S/N), was 0.078 μg/ml.

The method was applied to incurred dog plasma samples, and Fig. 4 shows the chromatogram obtained for blank dog plasma, a dog plasma spiked

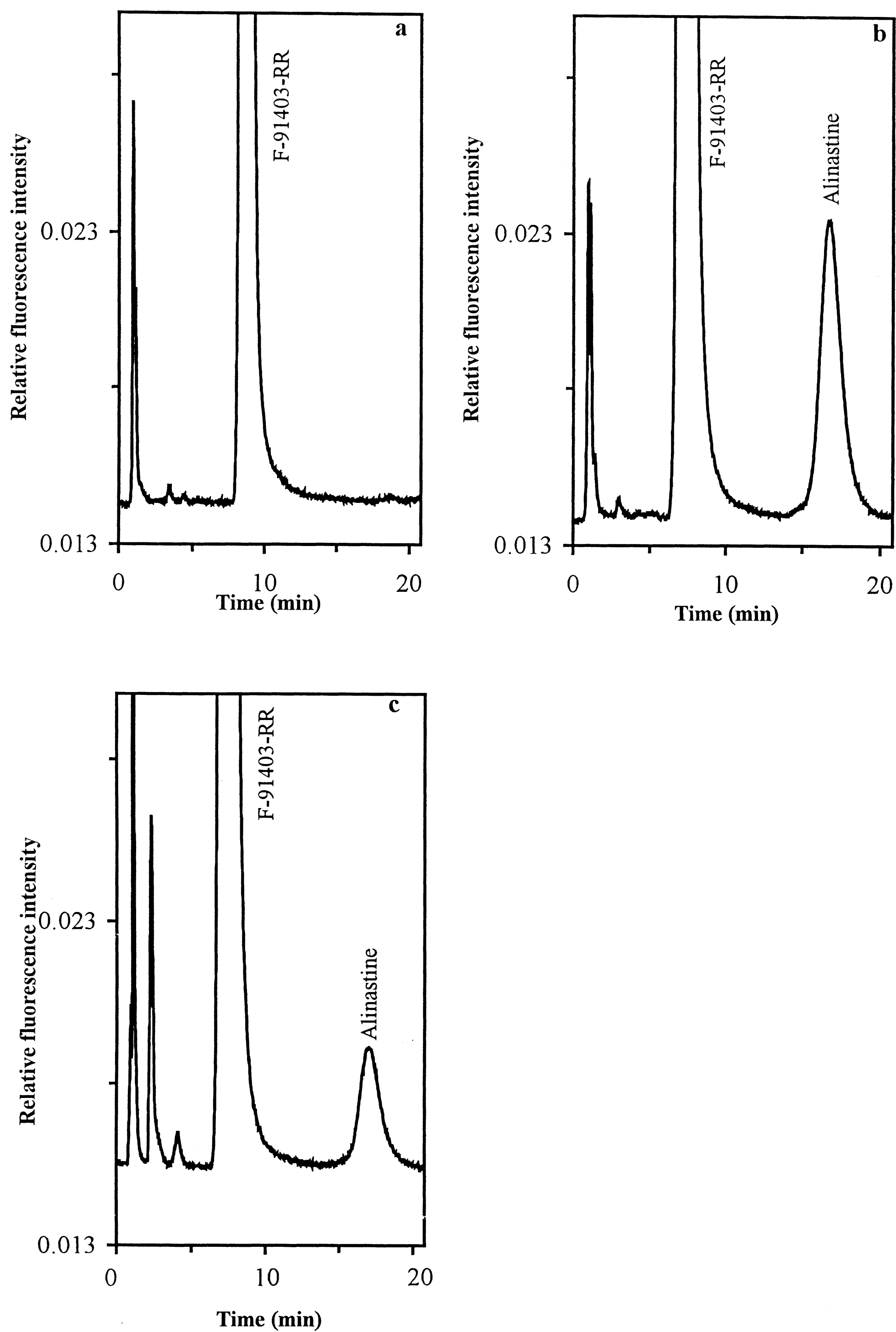

Fig. 4. Chromatograms corresponding to: (a) blank dog plasma, (b) dog plasma spiked with 0.1 μg/ml alinastine and (c) dog plasma collected after 28 days of alinastine oral treatment at 8 mg/kg·day.

Table 3
Recovery, within-day and between-day reproducibility for the assay of alinastine in dog plasma

	Day 1	Day 2	Day 3	Between-day repeatability
0.1 μg/ml				
Mean (μg/ml)	0.0543	0.0625	0.0614	0.0597
SD (μg/ml)	0.0018	0.0022	0.0022	0.0039
CV (%)	3.3	3.4	3.5	6.3
Mean recovery (%)	54.3	62.5	61.4	59.7
n	5	2	2	6
0.5 μg/ml				
Mean (μg/ml)	0.316	0.3467	0.3428	0.333
SD (μg/ml)	0.018	0.0025	0.0071	0.019
CV (%)	5.7	0.71	2.3	5.6
Mean recovery (%)	63.3	69.3	68.6	66.5
n	5	2	2	6
2 μg/ml				
Mean (μg/ml)	1.293	1.423	1.36	1.351
SD (μg/ml)	0.088	0.013	0.12	0.094
CV (%)	6.8	0.92	8.6	6.9
Mean recovery (%)	64.6	71.2	68.2	67.5
n	5	2	2	6
5 μg/ml				
Mean (μg/ml)	3.27	4.15	3.6217	3.70
SD (μg/ml)	0.10	0.37	0.0077	0.43
CV (%)	3.2	8.9	0.2	11.7
Mean recovery (%)	65.5	83.0	72.4	74.0
n	5	2	2	6

with 0.1 μl/ml alinastine and a dog plasma sample collected after 28 days of oral treatment at a dose of 8 mg alinastine/kg day. The amount of alinastine found in this real dog plasma sample was 0.19 μg/ml

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References

[1] R.D. McDowall, J. Chromatogr. 492 (1989) 3.
[2] R.D. McDowall, J.C. Pearce, G.S. Murkitt, J. Pharm. Biomed. Anal. 4 (1986) 3.
[3] T.R. Krishnan, I. Ibraham, J. Pharm. Biomed. Anal. 12 (1994) 287.
[4] N. Narasimhachari, J. Chromatogr. 225 (1981) 189.
[5] J.O. Svensson, B. Jonzon, J. Chromatogr. 599 (1990) 437.
[6] P.M. Kabra, J.H. Wall, N. Blanchart, Clin. Chem. 31 (1985) 1717.
[7] C.D. Gaitende, P.V. Pathak, J. Chromatogr. 528 (1990) 407.
[8] M. Dehnhard, H. Bernat-Bathelt, R. Claus, J. Chromatogr. 566 (1991) 101.
[9] N. Haagsma, E.R. Bathett, J.W. Engelsma, J. Chromatogr. 436 (1988) 73.
[10] M.A. Evenson, D.A. Engstrand, J. Anal. Toxicol. 13 (1989) 322.
[11] J. Doehl, T. Greilbrokk, J. Chromatogr. 529 (1990) 21.
[12] R. Hartley, M. Lucock, M. Becker, Y.J. Smith, W.I. Forsythe, J. Chromatogr. 377 (1986) 295.
[13] K.D. Setchell, C.H. Vestal, J. Lipid Res. 30 (1989) 1459.
[14] M. Casas, L.A. Berrueta, B. Gallo, F. Vicente, J. Pharm. Biomed. Anal. 12 (1994) 287.
[15] O. Suzuki, T. Kumazawa, H. Seno, H. Hattori, Med. Sci. Law 29 (1989) 243.
[16] M. Mazhar, S.R. Binder, J. Chromatogr. 497 (1989) 201.
[17] B.K. Logan, D.T. Stafford, I.R. Tebbet, C.M. Moore, J. Anal. Toxicol. 14 (1990) 154.

[18] X.-H. Chen, J. Wijsbeek, J.-P. Franke, R.A. De Zzeeuw, J. Forensic Sci. 37 (1992) 61.
[19] X.-H. Chen, J. Wijsbeek, J.-P. Franke, R.A. De Zzeeuw, J. Anal. Toxicol. 16 (1992) 351.
[20] X.-H. Chen, K. Ensing, J. Wijsbeek, J.-P. Franke, R.A. De Zzeeuw, J. Chromatogr. 16 (1992) 351.
[21] Ph. Hubert, P. Chiap, J. Crommen, J. Pharm. Biomed. Anal. 9 (1991) 877.
[22] Ph. Hubert, P. Chiap, J. Crommen, J. Pharm. Biomed. Anal. 9 (1991) 883.
[23] Ph. Hubert, J. Crommen, J. Liq. Chromatogr. 13 (1990) 3891.