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ANALYSIS OF CEPHALEXIN FROM CANINE SKIN BIOPSY BY LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET–VISIBLE PHOTODIODE-ARRAY DETECTION

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SUMMARY

A sensitive and selective ion-paired liquid chromatographic method with UV–VIS photodiode-array detection was developed to measure cephalexin in skin biopsy samples. The method involved a sonication of minced canine skin with ethanol–acetonitrile–water (30:20:50, v/v/v) and ultrafiltration of received extract through 10 000 daltons. Separation of cephalexin from other components was by liquid chromatography using a reversed-phase column which was eluted with an ion-paired acetonitrile–water solution. Detection was achieved with a UV–VIS photodiode-array detector scanning from 230 to 320 nm. Cephalexin in the eluate was quantitated at its wavelength maximum of 260 nm. The evaluation of chromatographic peak homogeneity was performed by absorbance ratios, contour maps, first-derivative spectra and a three-dimensional spectrochromatogram. Additionally, the cephalexin peak identity was confirmed by liquid chromatography–mass spectrometry.

INTRODUCTION

Cephalexin belongs to the cephalosporin, β-lactam group of antibiotics, with its structure (Fig. 1) incorporating the 7-aminocephalosporanic acid nucleus. Cephalosporin antibiotics are being used increasingly in veterinary medicine for treating bacterial infections [1].

In recent years, a considerable number of high-performance liquid chromato-

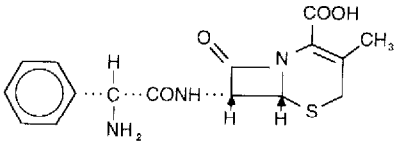

Fig. 1. Chemical structure of cephalexin.

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graphic (HPLC) procedures have been reported for the determination of cephalosporin antibiotics in body fluids [2]. Among them are a few reports concerning liquid chromatographic (LC) determination of cephalexin in serum and plasma with UV detection [3,4] and fluorometric detection [5]. Analysis of this antibiotic in canine skin or other tissues has not been reported. This manuscript reports a method which was devised to measure cephalexin in canine patients undergoing treatment for skin diseases.

Advantages of the photodiode-array detector over the photometric detector (UV–VIS) has been well documented in recent reports [6–9]. The ability of obtaining multi-wavelength in addition to single-wavelength chromatograms, which provides the ability to evaluate chromatographic peak homogeneity [10], is especially valuable.

This paper describes the analysis of cephalexin from canine skin biopsy samples by LC and UV–VIS photodiode-array detection. Due to the small sample size, a microbore (millibore) column was used to reach the higher mass sensitivities.

EXPERIMENTAL

Materials and reagents

All reagents were of analytical grade. Acetonitrile, methanol and phosphoric acid were HPLC grade (Fisher Scientific, Raleigh, NC, U.S.A.). Ion-pairing reagent, dodecanosulfonate-S12, was obtained from Regis (Morton Grove, IL, U.S.A.). Cephalexin base was supplied by Sigma (St. Louis, MO, U.S.A.). A 1 mg/ml stock solution of cephalexin was prepared in acetonitrile–ethanol–water (20:30:50). The working solution (1 μg/ml) was prepared daily from the stock solution.

The microseparation system, Centricon-10, molecular mass cut-off filter of 10 000 daltons was supplied by Amicon (Danvers, MA, U.S.A.). HPLC-grade water was obtained from a Model 1000 Hydro ultrapure water system of Hydro Services and Supplies (Research Triangle Park, NC, U.S.A.).

Sample preparation procedures

Skin biopsy samples were minced using a No. 10 Bard-Parker scalpel blade and weighed. Acetonitrile–ethanol–water (20:30:50) was added to 100–200 mg minced skin to a total volume of 500 μl and ultrasonicated for 30 min. The extract was transferred to a microseparation system and centrifuged for 20 min at 2677 *g* in a fixed-angle rotor. A 50-μl aliquot of colorless filtrate was injected into an HPLC unit with a photodiode-array detector.

The liquid chromatographic–mass spectrometric (LC–MS) confirmation of cephalexin peak identity was conducted on collected samples from the skin biopsy extracts and injected directly to the thermospray interphase (loop).

Instrumentation

The liquid chromatograph consisted of W600 multi-solvent delivery system with U6K injector and temperature control accessory set at 55 °C. This was cou-

pled to a 990 photodiode-array detector operated at 230–320 nm and 0.01 or 0.02 a.u.f.s. Chromatograms were recorded on 990 plotter (Waters Chromatography Division, Milford, MA, U.S.A.).

The instrument used for LC–MS consisted of a Model 510 pump and U6K injector (Waters) coupled to a Finnigan-MAT (San Jose, CA, U.S.A.) 4500 mass spectrometer with a Vestec thermospray (Vestec, Houston, TX, U.S.A.).

LC with UV–VIS photodiode-array detector

LC was performed in reversed-phase mode with dodecanosulfonate ions in the mobile phase to act as a modifier of the retention of cephalexin. The mobile phase consisted of 15% (v/v) acetonitrile in water containing 0.005 *M* dodecanosulfonate, 0.5% (v/v) of 85% phosphoric acid, and 0.5% (v/v) triethylamine. The column used for analysis was a microbore Brownlee phenyl Spheri-5 analytical cartridge, 22 cm × 2.1 mm I.D. in a 22-cm MPLC holder (Sci-Con, Winter Park, FL, U.S.A.). A mobile phase flow-rate was adjusted between 0.3 and 0.5 ml/min to give retention times for cephalexin of approximately 10 and 12 min. Peak-height or -area measurements were performed manually.

Liquid chromatography–mass spectrometry

Thermospray spectra were acquired using a mobile phase of methanol–0.1 *M* ammonium acetate (50:50) at a flow-rate of 1.2 ml/min. The thermospray interface was operated with a source temperature of 280 °C and a vaporizer temperature (T^2) of 190 °C. The interface was operated in the positive-ion detection mode with the filament on (0.2 mA at 1000 eV) to enhance chemical ionization. The instrument was operated in the multiple-ion detection mode scanning m/z 304 and m/z 348 in 0.5 s.

RESULTS AND DISCUSSION

Previous works concerning the determination of β-lactam antibiotics in serum and tissue extracts showed the usefulness of the microseparation system in removing macromolecules and eliminating the need for time-consuming solvent extraction for determination of ampicillin [11] and other penicillins [12] in serum and cefazolin in serum and tissues [13].

Based on earlier studies with these antibiotics the Spheri-5 phenyl column was determined to be optimum for HPLC separation. Due to the small volume of skin biopsy samples, the microbore Spheri-5 phenyl column was chosen for analysis of cephalexin.

Various elution systems consisting of two ion-pairing reagents were investigated: octanesulfonate and dodecanesulfonate and different amounts of acetonitrile and phosphoric acid. The best separation of cephalexin from other compounds was obtained with the mobile phase described in Experimental.

Cephalexin, like many β-lactam antibiotics, exhibits significant binding to serum [14] and tissue proteins. Several solutions were tested for their ability to free cephalexin from serum and tissue protein binding. Based on recovery of ce-

phalexin for both matrices, ethanol–acetonitrile–water (30:20:50) was chosen. The results concerning analysis of cephalexin in serum will be presented elsewhere.

The study on linearity of photodiode-array detector response was performed by injecting a cephalexin standard containing increasing amounts from 10 to 150 ng. Each amount was analyzed three times. The relationship between peak area and concentration of aqueous cephalexin solution was linear over 5 μg/ml of extract, which corresponded with approximately 10–25 μg/g of skin. The correlation coefficient was 0.9991 with $n=5$.

Fig. 2A depicts a chromatogram of 100 ng cephalexin standard solution (0–20 min), blank (20–40 min) and skin biopsy extract spiked with 100 ng (40–60 min). Fig. 2B shows the contour diagram of this chromatogram. Analysis of blank skin biopsy extract revealed only a single, very low level of interference under the cephalexin peak (50 μl sample). However, the contour diagram did not show any contours in the analytical window for cephalexin in the blank extract. The con-

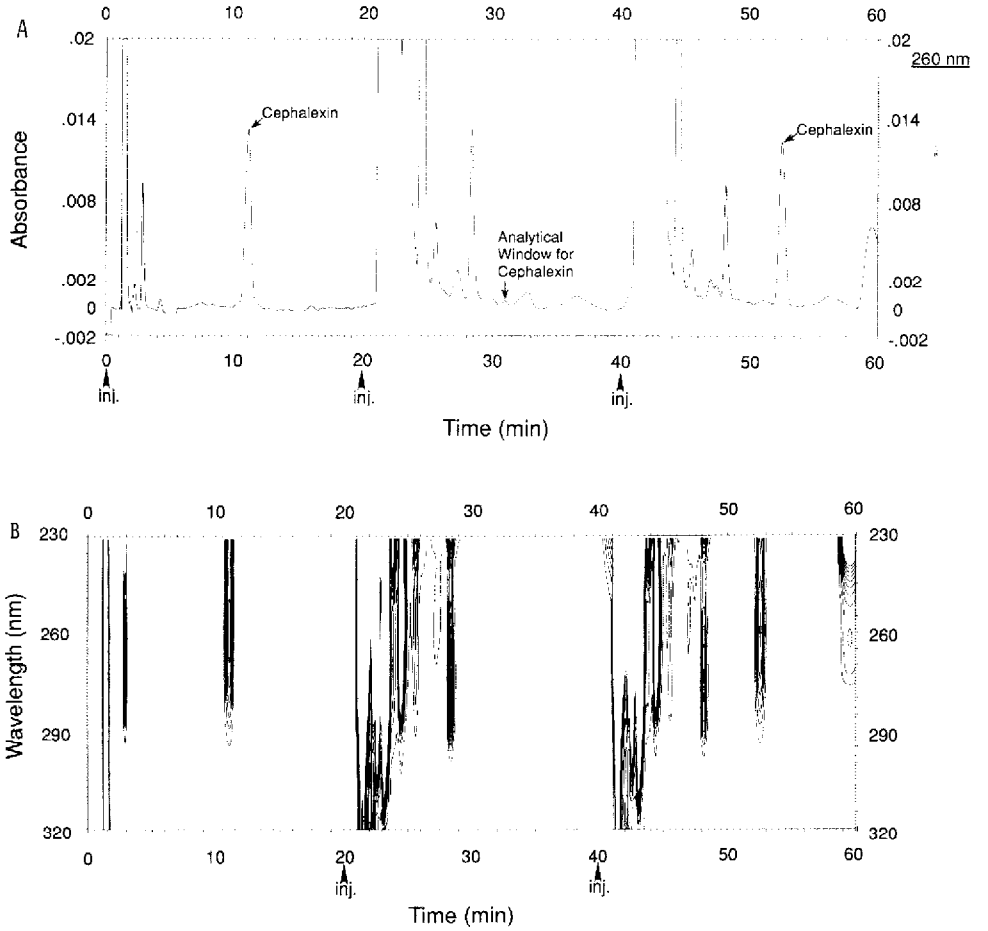

Fig. 2. (A) Chromatogram of 100 ng cephalexin standard (0–20 min), blank canine skin biopsy extract (20–40 min) and extract spiked with 100 ng cephalexin (40–60 min). (B) Contour plot of 100 ng cephalexin standard, blank and canine skin biopsy extract spiked with 100 ng cephalexin.

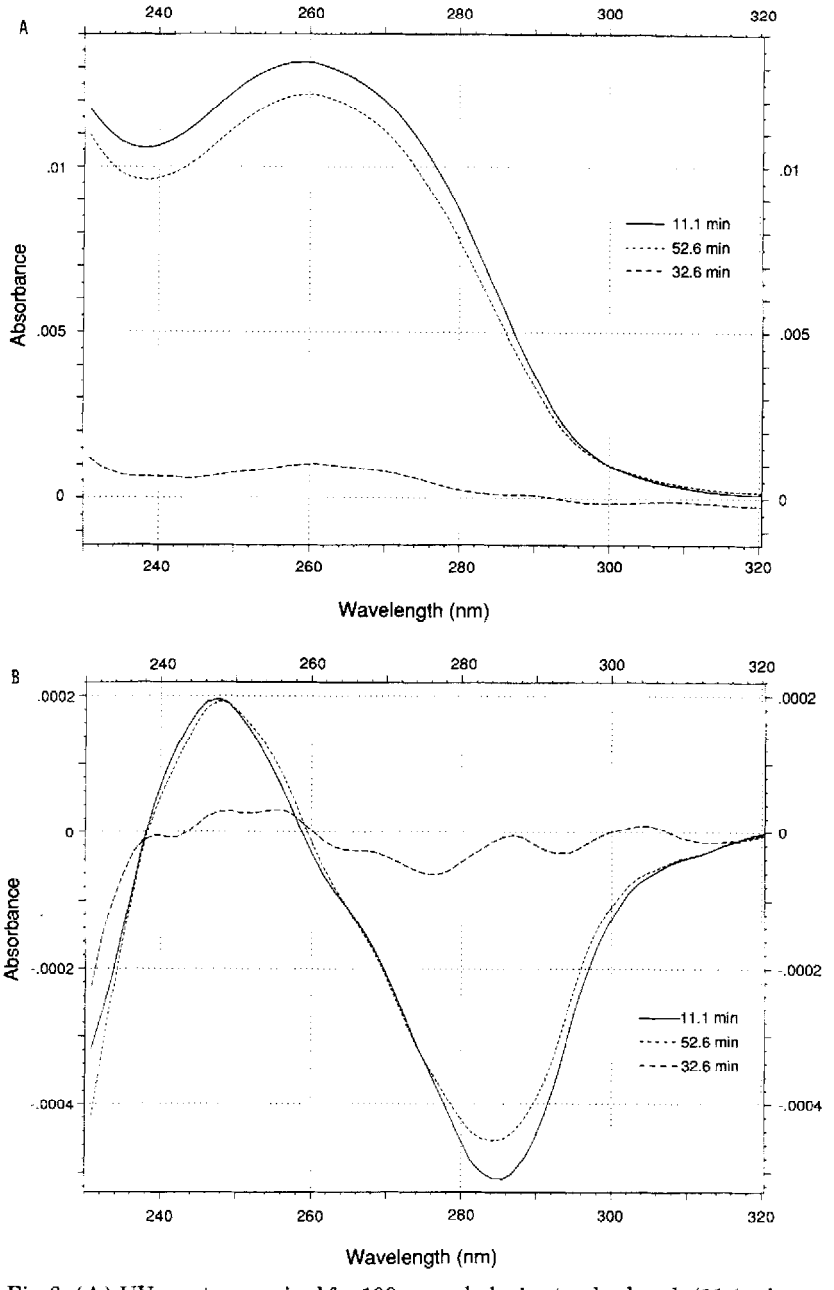

Fig. 3. (A) UV spectra acquired for 100 ng cephalexin standard peak (11.1 min running time), canine skin biopsy extract spiked with 100 ng cephalexin (52.6 min running time; injected at 40 min; 12.6 min after injection) and the analytical window for cephalexin in the blank extract (32.6 min running time; injected at 20 min; 12.6 min after injection). (——) Standard; (....) spiked sample; (----) blank sample. (B) First derivatives of spectra introduced in (A).

tours for cephalexin in standard and the spiked extract of skin biopsy were symmetric and identical which indicated the absence of interfering compounds. The spectral curves of absorbance for cephalexin in standard (11.1 min after injection) and spiked extract (52.6 min running time; 12.6 min after injection) showed good correlation with maximum at 260 nm (Fig. 3A). Additionally, the spectral curve was acquired at the cephalexin retention time (32.6 min running time; 12.6 min after injection) on the blank chromatogram. This curve showed some absor-

TABLE I

REPEATABILITY OF CEPHALEXIN ANALYSIS USING SPIKED SKIN EXTRACTS WITH 2 μg/ml CEPHALEXIN

Parameter	Value
Number of repeats (n)	9
Range (μg/ml)	1.72–1.87
Mean (x)	1.82
Standard deviation	0.0454
Coefficient of variation (%)	2.5
Recovery (%)	91.0

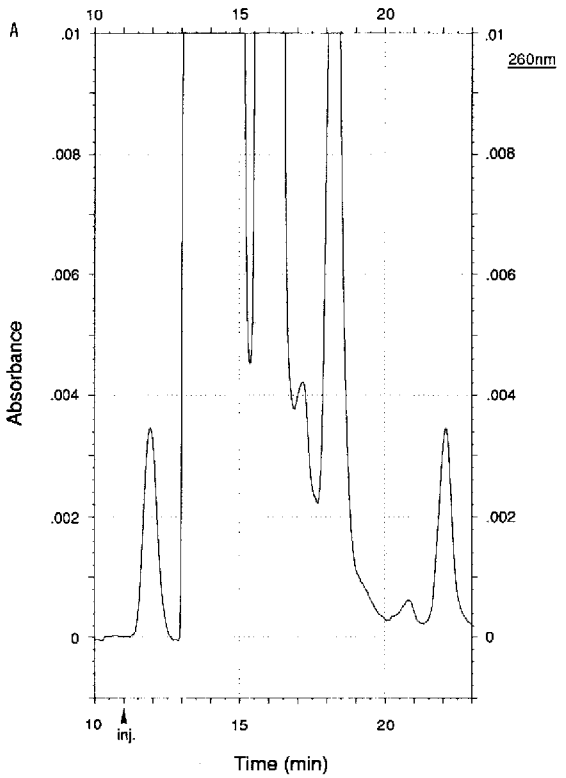

Fig. 4.

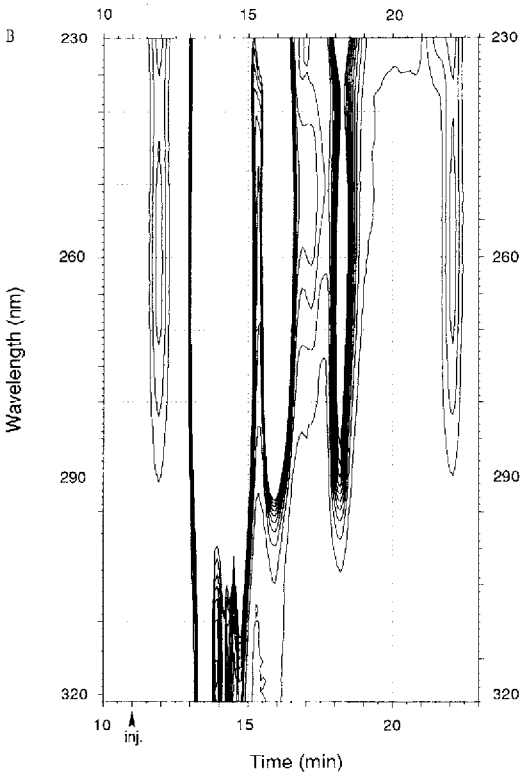

Fig. 4. (A) Part of the chromatogram of standard cephalexin solution injected at time 0 (not shown) and true canine skin biopsy extract with cephalexin, injected at 11 min running time. (B) Contour plot of chromatogram in (A).

bance at 260 nm as well. Fig. 3B depicts the first derivative (dA/dt) of these curves. The derivative plot proved the identity of cephalexin in standard and skin biopsy extract and showed that a curve obtained at 32.6 min of analysis had different spectral characteristics than cephalexin. The derivative plot confirmed that the peak on the blank chromatogram could easily be distinguished from the cephalexin peak. However, larger amounts of extract ($>50\,\mu l$) should be applied cautiously.

Table I shows the accuracy and precision for cephalexin determination in skin biopsy samples. The coefficient of variation determined from peak heights was 2.5%. The detection limit (defined as a peak at least three times the height of baseline noise) was approximately 100 ng/g of skin (10–20 ng/ml of extract).

A typical separation of cephalexin from true skin biopsy extract is shown in Fig. 4A. A standard solution containing 25 ng cephalexin had a retention time of 11.9 min. At 11 min following injection of the standard solution a second injection containing true skin biopsy extract was performed. Fig. 4B depicts a contour diagram for that part of chromatogram (shown in Fig. 4A). Comparison of the contours for cephalexin standard and cephalexin in the biopsy sample showed almost identical symmetric plots except in the range 230–238 nm. However, this

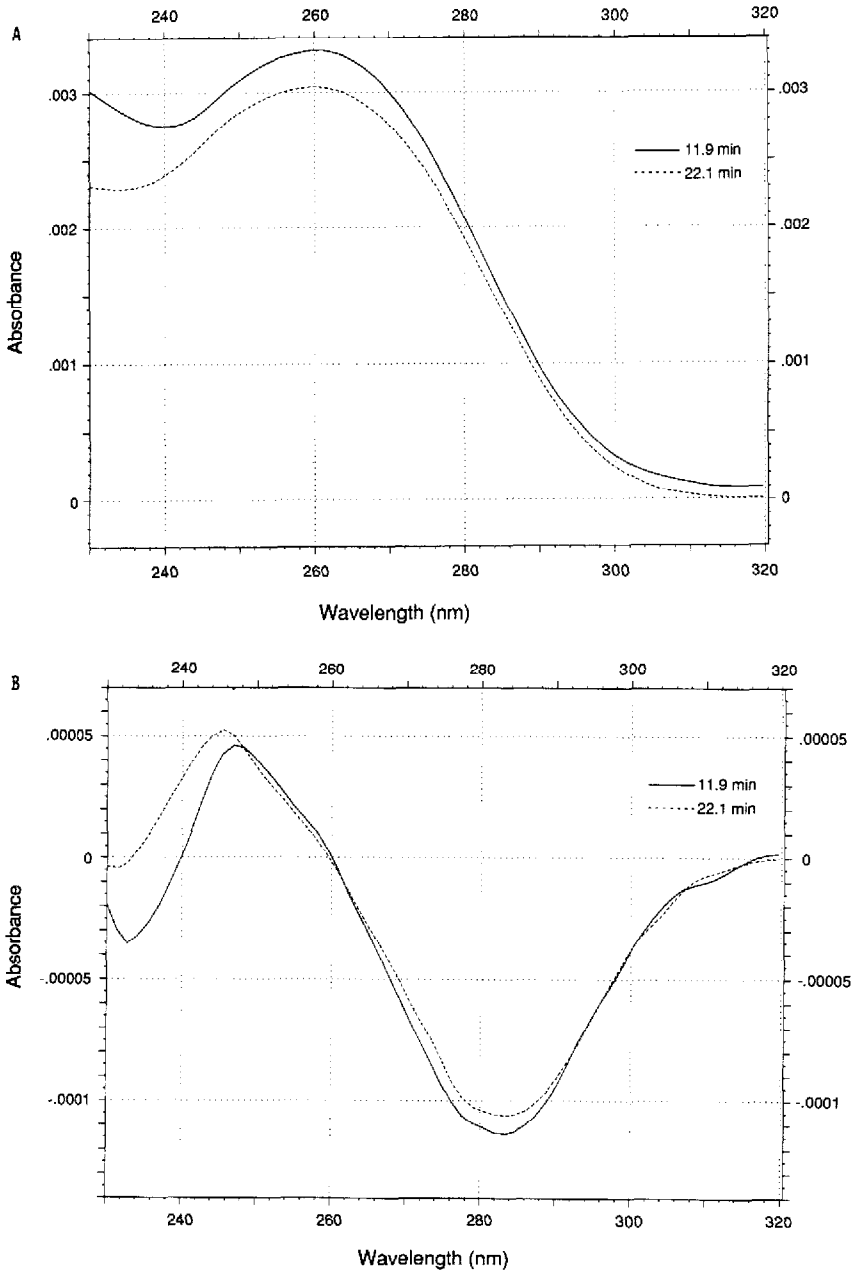

Fig. 5. (A) Comparison of spectra acquired at cephalexin standard peak at 11.9 min running time and cephalexin peak in true canine skin biopsy extract injected at 11 min running time and acquired at 11.1 min after injection. (——) Standard; (----) true sample. (B) First derivative of spectra introduced in (A). (——) Standard; (----) sample.

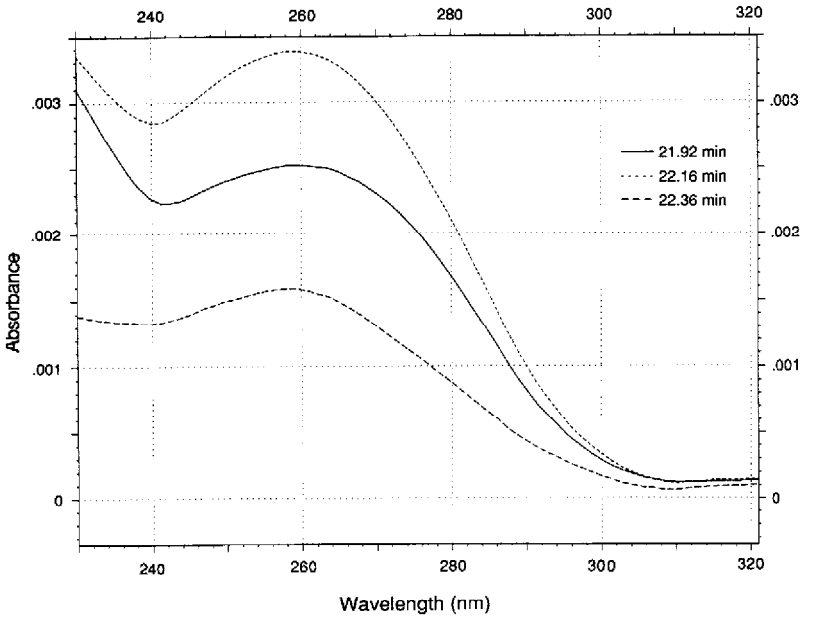

Fig. 6. Spectra of cephalexin peak acquired automatically at the upslope (21.92 min running time), apex (22.16 min running time) and downslope (22.36 min running time) from a canine subject. Injection appeared at 11 min running time.

does not present a problem since analyses are performed at 260 nm. The spectral curves of cephalexin absorbance and their first derivatives present good confirmation of cephalexin peak identity. Moreover, it is clear that overlapping peaks (Fig. 5A and B) do not occur at the wavelength that measurements are made (260 nm).

Additionally, to examine the purity of the cephalexin peak in true skin biopsy sample, three spectra were acquired over it: one on the upslope, one on the apex and one on the downslope. The three spectra are shown in Fig. 6. The differences between these spectra are only in amplitude and not in maxima or minima. This indicates that the cephalexin peak shows no coeluting interferences [6].

During analysis of cephalexin from skin biopsy samples a significant shift in cephalexin retention time (up to 2 min) was observed, probably due to complex sample matrix. Therefore an LC–MS confirmation was performed on cephalexin extracts from true skin biopsy extracts.

LC–MS was performed on 100 ng cephalexin standard and cephalexin collected from true canine skin biopsy extracts (approximately 300 ng). Results were obtained using the thermospray filament mode of ionization and positive-ion detection. The $[M+H]^+$ ion at m/z 348 and the $[M+H-CO_2]^+$ ion at m/z 304 were monitored for cephalexin to verify their presence in the biopsy extract. The HPLC–MS analysis of the extract exhibited a signal for these ions, at the same intensity ratio (intensity 348/intensity 304 of 1/8.7) as observed for the standard solution of cephalexin.

CONCLUSION

An original method for the determination of cephalexin in skin biopsy has been developed which provides a sensitive and selective analytical procedure for use in clinical studies.

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