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Original research paper

Rapid and sensitive HPLC method for the estimation of doxorubicin in dog blood – The silver nitrate artifact

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A rapid and sensitive high performance liquid chromatographic (HPLC) assay utilizing fluorimetric detection (excitation at 480 nm, emission at 560 nm) for the determination of doxorubicin in dog blood was developed and validated. Treatment of blood samples containing doxorubicin with $AgNO_3$ (as protein precipitant) resulted in appearance of an additional peak in the chromatogram of doxorubicin at 11.5 min along with the parent peak (t_R = 5.5 min). The latter peak was not found when treated with other protein precipitants such as trichloroacetic acid and methanol. Construction of a calibration curve based on the area of the first peak alone did not result in linearity of the curve. However, summation of areas of both peaks resulted in a curve with good linearity and coefficient of determination (R^2 = 0.9985). Appearance of the second peak may be due to the interaction of doxorubicin with cellular components of blood in the presence of $AgNO_3$ leading to the formation of a complex with reduced polarity. Analysis of the quality control samples showed good accuracy (96.7–100.42) and precision (RSD = 2.6–5.7%). The proposed method could be advantageous in estimation of doxorubicin incorporated into targeted delivery systems that concentrate in blood cells and quantify the absolute blood concentration of doxorubicin.

Keywords: doxorubicin, dog blood, dog plasma, silver nitrate, HPLC

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Doxorubicin is an anthracycline antibiotic with potent antineoplastic properties effective against a broad spectrum of malignancies such as non-Hodgkin's lymphoma, acute lymphoblastic leukaemia, breast carcinoma and several other types of cancers. The mechanism of cytotoxicity involves specific intercalation of the planar anthracycline nucleus of doxorubicin to the DNA double helix, resulting in prevention of further DNA replication (1). One of the major drawbacks in chemotherapy with doxorubicin is its cardiotoxicity (2). Pharmaceutical scientists have made several attempts to improve the the-

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rapeutic index of doxorubicin and overcome its adverse effects. As a result, a variety of drug delivery systems containing doxorubicin were developed, such as microspheres, liposomes, nanoparticles, antibodies and polyaminoacids.

Several methods were previously reported for quantification of doxorubicin in biological fluids and tissues. Techniques such as radioimmunoassay for determination of doxorubicin in plasma and urine (3), voltammetry for urine (4, 5), high performance liquid chromatography (HPLC) for rat lymph and gall (6), rat plasma and tissues (7), rat serum, tissues (8) and bile (9), human plasma of cancer patients (10) and in blood (11), and spectrofluorimetry for rabbit serum (12) and rat whole blood, plasma and tissues (13) were used. Many of the above mentioned HPLC methods used extraction procedures involving organic solvents such as chloroform or dichloromethane. Such procedures are cumbersome and involve risks of incomplete drug extraction, loss of drug during the transfer of contents and also high volatility of the solvent used for extraction. Hence, a simple HPLC method, which avoids the above risk factors, would be advantageous for rapid determination of doxorubicin in biological samples.

All the above mentioned HPLC techniques were used to determine doxorubicin concentrations in plasma, serum or lymph. No method was reported for whole blood, which includes combined estimation in plasma and blood cells.

As doxorubicin is a cytotoxic agent, there is a great possibility of its accumulation in blood cells, especially when the drug is incorporated or bound to targeted delivery systems such as nanoparticles or liposomes. Under these conditions, estimation of doxorubicin in plasma alone may result in underestimation of drugs circulating in the blood. Hence, estimation of doxorubicin in whole blood may be meaningful since it provides a true picture of the drug concentration in plasma as well as in blood cells.

In the present investigation, an attempt was made to develop a simple, rapid and sensitive HPLC method for the estimation of doxorubicin in dog blood, and relevant analytical parameters were established.

EXPERIMENTAL

Chemicals and reagents

Doxorubicin hydrochloride (DH) was a gift sample from RPG Life Sciences (India). HPLC grade methanol and acetonitrile were purchased from J. T. Baker (USA).

All other chemicals and reagents used in the study were of analytical grade. Water used for analysis was purified through the MilliQ water system (Millipore, USA).

Animals

Blood samples were collected from three male Mongrel dogs weighing 18–20 kg. The dogs were housed in an animal cage and were fasted overnight before the collection of blood samples**.** The dogs had access to water *ad libitum*. The dogs were cared and the blood was collected as per the ethical standards of the institution.

Standard solutions of doxorubicin

An accurate amount of doxorubicin hydrochloride was weighed in order to prepare 1 mg mL^{-1} stock standard solution in methanol. The stock solution was stored at 5 °C when not in use.

Mobile phase

The buffer, pH 4.0, was prepared from 10 mmol L^{-1} ammonium hydrogen phosphate solution, to which 5 mL trimethyl amine was added. The pH of the solution was adjusted to 4.0 with orthophosphoric acid.

The mobile phase was a mixture of buffer pH 4.0/acetonitrile/methanol (60:24:16, *V/V/V*), sonicated for 10 min (Toshniwal ultrasonic cleaner, India) and filtered through 0.22 μm filter.

Reagents

Aqueous silver nitrate solution (30% *m/V*) was prepared by dissolving silver nitrate in purified water. 100 μL of the reagent was used as protein precipitant.

Aqueous trichloroacetic acid (TCA) (10% *m/V*) was prepared by dissolving trichloroacetic acid in purified water. 100 μL of the reagent was used as protein precipitant.

DNA solution (1 mg mL^{-1}) was prepared by dissolving Salmon sperm DNA as model DNA (Hi-Media, India) in purified water. 100 μL of the reagent was used for the interaction studies.

Instrumentation

The HPLC system (Waters, Milford, USA) used in this study consists of a Waters 2690 separations module (comprising a pump, a refrigerated auto sampler with a 100 μL loop) and a Shimadzu RF-10 AXL fluorescence detector (Shimadzu Corporation, Japan). The detector was set at 480 nm and 560 nm (excitation and emission wavelengths, respectively). The detector was set at gain 3 and sensitivity 1. Chromatographic separation was performed on a 150 x 4.6 mm i.d. reversed-phase column (Inertsil ODS-2, GL Sciences, Japan) of 5 μ particle size. Separation of analytes was performed at a flow rate of 1.3 mL^{-1} min^{-1}, and typical back pressure was 12.67 MPa.

Blood samples

Standard solution of doxorubicin hydrochloride (DH) was added to 400 μL dog whole blood (containing EDTA) in a glass tube in respective amounts to obtain the final concentrations of 5, 10, 40, 80, 160, 240 and 480 ng mL^{-1} of DH. To this mixtures 100 μL of 30% $AgNO_3$ was added. The contents were vortexed for 1 min and 5 mL methanol was added. The mixture was extracted (Cole multipulse vortexer, Glas-Col, USA) for 10 min and centrifuged at 2000 rpm for 10 min at 20 °C. The supernatant was decanted into another glass tube and evaporated to dryness at 60 °C under a stream of nitrogen. The dried residue was reconstituted with 200 μL methanol and centrifuged at 15000 rpm for 10 min. Clear supernatant was collected in HPLC vials and loaded onto the HPLC system.

Plasma used in the study was isolated from the whole blood containing EDTA by centrifugation (Sigma 3K30 refrigerated High speed centrifuge, Sigma Instruments, Germany) at 3000 rpm for 5 min.

Method validation

During method validation, LOD and LLOQ values and linearity were established from the chromatograms based on three separate runs of freshly prepared calibration standards. LOD and LLOQ were determined upon signal-to-noise approach, by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified with precision. The analyte response at the LOD should be at least 3 times and at the LLOQ it should be at least 5 times the response compared to the blank response.

$$\text{LOD} = \frac{\text{concentration of injected sample x 3}}{\text{S/N ratio of the injected sample}} \qquad \text{LLOQ} = \frac{\text{concentration of injected sample x 5}}{\text{S/N ratio of the injected sample}}$$

Accuracy of the method was tested by subjecting known amounts of doxorubicin to recovery studies in six replicates in whole blood.

Stability

Stability of doxorubicin in dog whole blood samples was determined at two different temperatures. DH was incubated in dog whole blood at concentrations of 75, 150 and 225 ng mL^{-1} at 5 °C for different time intervals ranging from 0 to 8 h, and at room temperature (28 °C) for up to 3 days and assayed. Determinations were made in triplicate.

Results and discussion

A rapid and sensitive HPLC method was developed for the estimation of doxorubicin in dog blood, which avoids extraction with non polar organic solvents such as chloroform, dichloromethane (DCM), *etc*. Doxorubicin is a basic molecule (pK_a = 8.22) (1), and for better extraction into organic solvents such as chloroform or DCM, the sample pH should be adjusted to alkaline range (pH > 8.0). But, doxorubicin is unstable at alkaline conditions (at about pH 9.0 it shows an indicator-like effect) (1). Unlike the reported methods which used pH 9.2 borate buffer for extraction of doxorubicin into organic solvents (11, 14), in the present method, the pH of the buffer used for mobile phase preparation was adjusted to 4.0, and hence can avoid the pH-dependent degradation problems. As doxorubicin is a cytotoxic agent, after administration into the body, it could be expected to penetrate and concentrate in blood cells. Hence its determination in plasma or serum alone by neglecting the blood cells may lead to underestimation of the concentration. Estimation in whole blood becomes important when the drug is incorporated into delivery systems for purposes such as controlled and targeted delivery, since the delivery system may concentrate in cells rather than circulate in plasma. Hence a new method was developed for the determination of doxorubicin in dog whole blood.

Figs. 1a–c show typical chromatograms of the doxorubicin standard retention time of 5.5 min, dog blank blood extract and doxorubicin-spiked blood extract. No interfering peaks appeared in the chromatogram of the blood extract at the retention time of doxorubicin.

Protein precipitation of blood samples was carried out by addition of aqueous $AgNO_3$ solution (Fig. 2a). Interestingly, this process resulted in appearance of a second peak with retention time of 11.5 min in the chromatogram of the blood sample spiked with doxorubicin.

To understand the cause of the appearance of the second peak in the chromatogram of doxorubicin, a series of experiments with various permutations and combinations, involving the influence of DNA (model DNA) and various protein precipitants were carried out and the results are recorded in Table I. The second peak (t_R = 11.5 min) did not appear in the chromatogram of doxorubicin when the protein precipitation of blood containing doxorubicin was carried out using other reagents such as TCA (Fig. 2b) and methanol. It was hypothesized that the intercalating nature of doxorubicin with cellular DNA might have resulted in a complex with reduced polarity, appearing as the second peak. However, the chromatograms of the model systems of doxorubicin incubated with

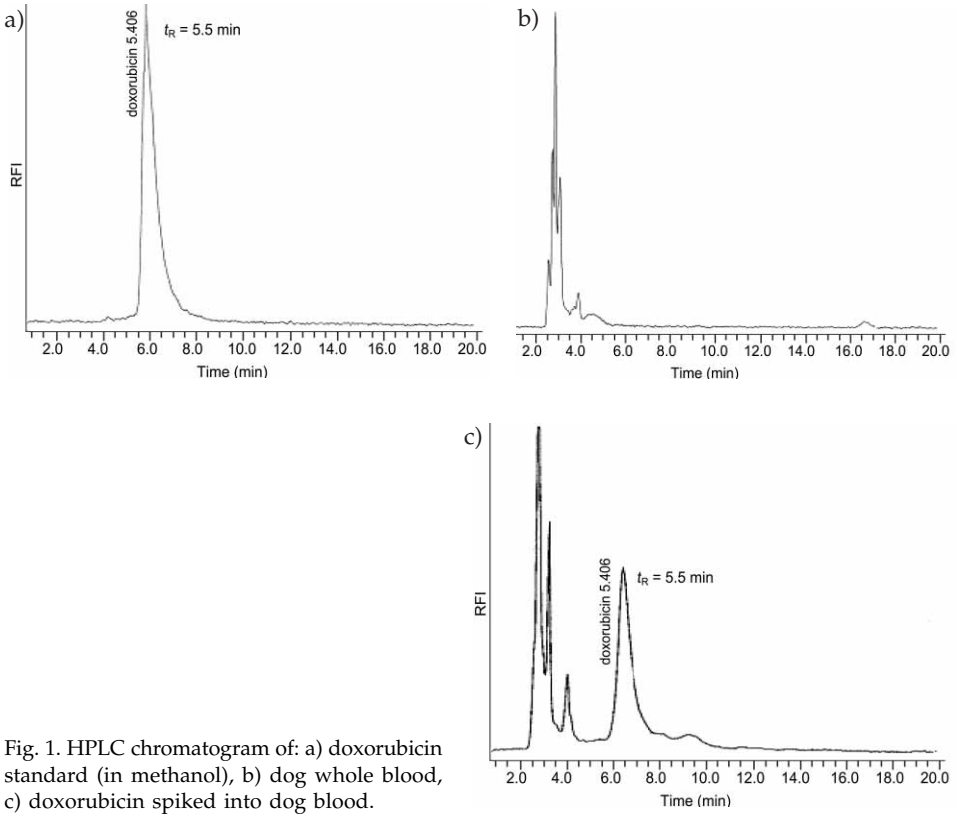

Fig. 1. HPLC chromatogram of: a) doxorubicin standard (in methanol), b) dog whole blood, c) doxorubicin spiked into dog blood.

$AgNO_3$ solution in the presence or in the absence of DNA did not result in the second peak, proving that neither $AgNO_3$ nor DNA alone is responsible for the appearance of the second peak, under *in vitro* conditions.

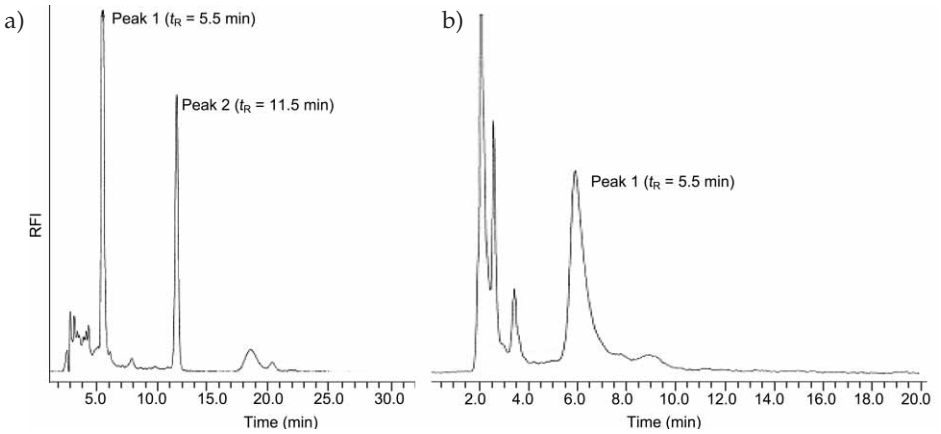

Fig. 2. Chromatogram of doxorubicin spiked into dog blood and treated with a protein precipitant: a) $AgNO_3$, b) trichloroacetic acid.

Table I. Appearance of doxorubicin in dog whole blood and plasma samples

Sample composition	t_{R1} (min)	t_{R2} (min)
Doxorubicin solution (Fig. 1a)	5.5	–
DNA solution	–	–
$AgNO_3$[a]	–	–
Doxorubicin + DNA	5.5	–
Doxorubicin + $AgNO_3$[a]	5.5	–
Whole blood + $AgNO_3$[a]	–	–
Whole blood + trichloroacetic acid[b]	–	–
Whole blood + methanol	–	–
Whole blood + doxorubicin (Fig. 1c)	5.5	–
Whole blood + doxorubicin + $AgNO_3$[a] (Fig. 2a)	5.5	11.5
Whole blood + doxorubicin + trichloroacetic acid[b] (Fig. 2b)	5.5	–
Whole blood + doxorubicin + methanol	5.5	–
Plasma + doxorubicin + $AgNO_3$[a] (Fig. 3a)	5.5	–
Haemolyzed plasma + doxorubicin + $AgNO_3$[a]	5.5	11.5
Plasma + doxorubicin + trichloroacetic acid[b] (Fig. 3b)	5.8	–
Plasma + doxorubicin + methanol (Fig. 3c)	5.5	–

[a] 30% aqueous solution
[b] 10% aqueous solution

Experiments were also conducted in dog plasma to check the appearance of the second peak. Plasma samples with or without doxorubicin were prepared in a similar way to that of whole blood samples, using three different protein precipitants, *i.e.*, $AgNO_3$, TCA and methanol. Unlike the whole blood samples, the chromatograms of plasma samples containing doxorubicin did not show the presence of the second peak (t_R = 11.5 min) with any of the above protein precipitants (Fig. 3). In order to check whether the second peak appears only in the presence of blood components, the doxorubicin was spiked into slightly haemolyzed plasma and treated individually with the mentioned three protein precipitants and subjected for HPLC. Interestingly, the second peak appeared in the chromatograms of haemolyzed plasma samples treated with $AgNO_3$ solution. A similar case could also be expected with tissues when $AgNO_3$ is used as protein precipitant. This strongly suggests that the second peak is due to the interaction of doxorubicin with cellular components in the presence of $AgNO_3$ solution. Hence this fact has to be noted and caution has to be exercised while using silver nitrate as protein precipitant for the treatment of whole blood or haemolyzed plasma samples in the estimation of doxorubicin in biological samples.

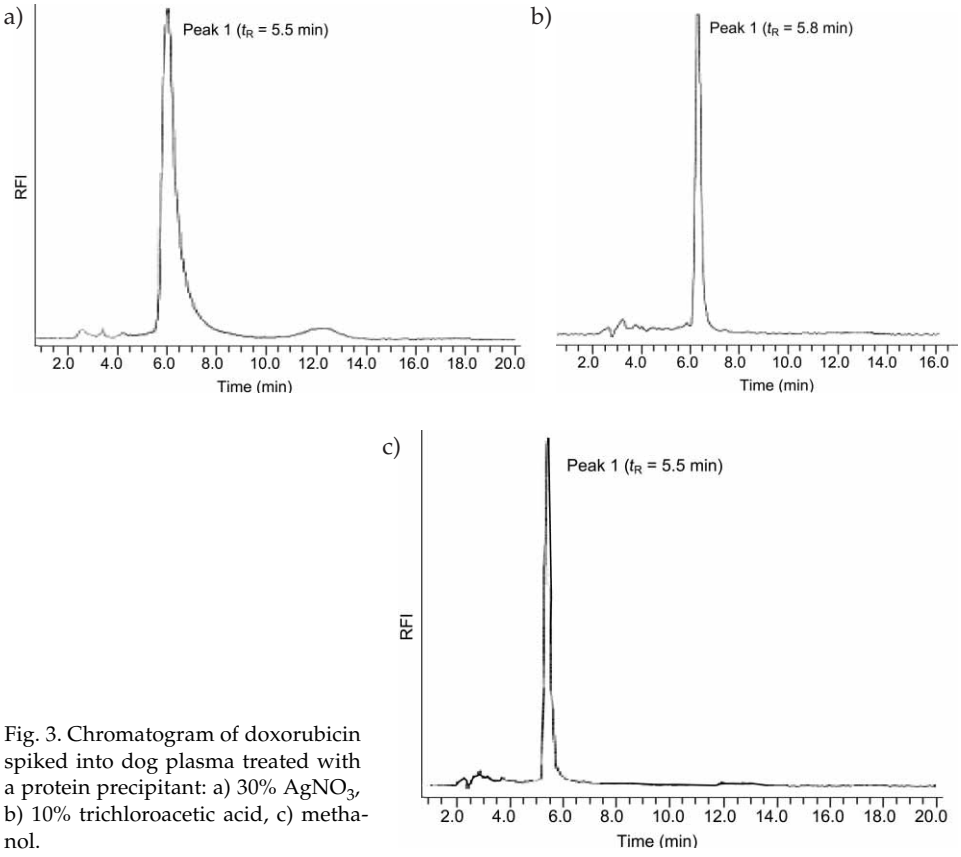

Fig. 3. Chromatogram of doxorubicin spiked into dog plasma treated with a protein precipitant: a) 30% $AgNO_3$, b) 10% trichloroacetic acid, c) methanol.

Table II. Accuracy and precision of HPLC determination of doxorubicin in dog whole blood

Theoretical concentration (ng mL^{-1})	Measured concentration, CI (ng mL^{-1})[a]	Accuracy (%)	Precision (%)[b]
75	72.55 ± 4.37	96.7	5.7
150	146.07 ± 6.60	97.4	4.3
225	225.86 ± 6.21	100.4	2.6

[a] CI – Confidence interval at $p = 0.05$.
[b] $n = 6$ replicates.

Table III. Stability of doxorubicin in dog blood

Theoretical concentration (ng mL^{-1})	Temperature (°C)	Time (days)	Confidence interval (ng mL^{-1})[a,b,c]
75	5		
		1	74.15 ± 7.73 (98.9)
		2	71.75 ± 8.76 (95.7)
		3	72.68 ± 7.76 (96.9)
150			
		1	148.59 ± 13.72 (99.1)
		2	146.89 ± 13.65 (97.9)
		3	143.80 ± 11.10 (95.9)
225			
		1	222.66 ± 14.34 (99.0)
		2	225.83 ± 12.74 (100.4)
		3	221.50 ± 13.62 (98.4)
	28	Time (h)	
75			
		0	72.34 ± 8.25 (96.5)
		4	73.37 ± 9.04 (97.8)
		8	72.18 ± 9.04 (96.2)
150			
		0	147.54 ± 12.35 (98.4)
		4	149.87 ± 12.04 (99.9)
		8	147.18 ± 12.04 (98.1)
225			
		0	221.63 ± 10.31 (98.5)
		4	222.04 ± 12.07 (98.7)
		8	224.08 ± 12.07 (99.6)

[a] Confidence interval at $p = 0.05$.
[b] $n = 3$ replicates.
[c] Recovery indicated in brackets.

Construction of a calibration curve of doxorubicin in dog blood treated with $AgNO_3$ as protein precipitant, and consideration of the area of the first peak alone (t_R = 5.5 min) did not result in a linear curve. However, summation of areas of both peaks (5.5 and 11.5 min) resulted in a calibration curve with excellent linearity and coefficient of determination, R^2, of 0.9985. The regression equation obtained is:

$$y = 40427x + 27319$$

The bias values of the standard curve were less than 7%, and the standard deviation values of three replicates were less than 0.5%. The lower limit of quantification, LOQ, of doxorubicin in blood was 12.38 ± 0.89 ng mL^{-1} with an RSD of 11.6%. The limit of detection (LOD) was found to be 3.52 ng mL^{-1}.

A good linearity and correlation coefficient of the calibration curve constructed by the summation areas of two peaks suggests the interaction of doxorubicin with the blood components in the presence of $AgNO_3$. This results in a complex with reduced polarity, eluting in the form of a second peak at 11.5 min. The presence of the second peak was also confirmed in the whole blood samples of dog, to which doxorubicin was administered by intravenous or intraperitoneal route (unpublished data).

Accuracy of the method was determined by subjecting known amounts of doxorubicin to recovery studies in dog blood (n = 6). Table II presents the results obtained. The concentrations selected for the assay were 75, 150 and 225 ng mL^{-1} and the percentage recovery was found to be 96.7 to 100.4%. Precision of the method was ascertained for six replicate assays at 75, 150 and 225 ng mL^{-1}: RSD ranged from 2.6 to 5.7%.

Results for doxorubicin stability in dog blood samples obtained are shown in Table III. The percent recovery of doxorubicin under the above storage conditions was found to be between 95.7 and 100.4%. No appreciable variation in the measured concentrations in dog blood samples following storage at 28 °C for up to 8 h, and at 5 °C for up to 3 days, indicate the stability of doxorubicin in dog whole blood.

CONCLUSIONS

The proposed HPLC method is simple, rapid and sensitive for the determination of doxorubicin in dog whole blood. It has been shown to be precise and capable of accurately quantifying the drug. It is concluded that the treatment of whole blood or haemolyzed plasma samples containing doxorubicin with $AgNO_3$ results in appearance of an additional peak along with the parent peak in the chromatogram. Hence, caution has to be exercised while using $AgNO_3$ for the treatment of whole blood or plasma samples for the estimation of doxorubicin.

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S A Ž E T A K

Brza i osjetljiva HPLC metoda za određivanje doksorubicina u krvi psa – Artifakt srebrovog nitrata

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Razvijena je i validirana brza i osjetljiva metoda visokotlačne tekućinske kromatografije (HPLC) s fluorimetrijskom detekcijom za određivanje doksorubicina u krvi psa. Nakon obrade uzoraka krvi koji sadrže doksorubicin s $AgNO_3$ (taložno sredstvo za proteine), uz osnovni signal (t_R = 5,5 min) pojavljuje se dodatni signal u kromatogramu doksorubicina na 11,5 min. Nikakav dodatni signal se ne pojavljuje ako se za taloženje proteina upotrijebe trikloroctena kiselina ili metanol. Međutim, u hemoliziranim uzorcima plazme kojima je dodan doksorubicin nakon obrade sa srebrovim nitratom pojavljuje se drugi signal. Kalibracijska krivulja s površinom ispod signala za samo prvi pik (t_R = 5,5 min) nije linearna. Međutim, suma površina ispod prvog (t_R = 5,5 min) i drugog signala (t_R = 11,5 min) daje linearnu zavisnost s korelacijskim koeficijentom R^2 = 0,9985. Pojava drugog signala mogla bi biti posljedicom interakcije doksorubicina sa staničnim komponentama u krvi u prisutnosti $AgNO_3$ te nastajanja kompleksa smanjene polarnosti. Površina ispod signala na kromatogramu određena na temelju fluorescencijske detekcije na 480 nm i 560 nm (valne duljine pobuđivanja, odnosno emisije) upotrebljena je za kvantitativnu analizu doksorubicina. Određivanje doksorubicina u krvi psa je jednostavno, precizno i točno. U svim eksperimentima, relativna standardna devijacija (RSD) najčešće je bila < 10%, a izmjerena i teorijska koncentracija razlikovala se za < 10%. Doksorubicin inkubiran s uzorcima krvi psa bio je stabilan najmanje 3 dana čuvan na 5 °C, odnosno najmanje 8 h čuvan na sobnoj temperaturi (28 °C). Predložena HPLC metoda mogla bi imati prednosti u određivanju doksorubicina u sustavima za isporuku lijekova ciljano u krvne stanice i kvantifikaciji ukupne koncentracije doksorubicina u krvi.

Ključne riječi: doksorubicin, krv psa, plazma psa, srebrov nitrat, HPLC

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