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Estimation of *In-vitro* Plasma Protein Binding of Ampicillin in Horses Based on Spectrophotometric Method

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ABSTRACT

Estimation of plasma protein binding (PPB) is of paramount importance in the pharmacokinetics characterization of drugs, as it can cause significant change in volume of distribution, clearance and half-life of the drug. Ampicillin (α-amino benzyl penicillin) is most commonly used drug in equine practice. This study was conducted to determine the extent of PPB of ampicillin in apparently healthy horses (n=6). A simple spectrophotometric method was applied for the determination of ampicillin at 320 nm wavelength, based on acid degradation product of penicillin at 75°C in presence of citrate buffer (pH 5.2) and traces of copper salt. In the study, it was observed that this method permits the detection of ampicillin to a level not beyond 1.0 µg/ml. Various concentrations of ampicillin (3.125, 6.25, 12.5, 25, 50, 100 µg/ml) were prepared in triplicate in pooled plasma collected from healthy animals. *In vitro* binding of ampicillin to plasma proteins was determined by employing the equilibrium dialysis technique. The study revealed that the plasma protein binding of ampicillin was to the extent of 12.8 ± 0.07 %. Binding capacity of ampicillin to plasma protein (β_i) and dissociation rate constant of protein-drug complex (K_β) in the present study were $0.34 \times 10^{-6} \pm 0.02 \times 10^{-6}$ mol.gm^{-1} and $0.003 \times 10^{-9} \pm 0.0003 \times 10^{-9}$ mol, respectively in horses. Hence, the study concluded that usage of spectrophotometric method helps in quick, cost effective and efficient results in estimation of PPB for ampicillin.

Keywords: Ampicillin, plasma protein binding, spectrophotometric method, horse

Since discovery, penicillin have been very effective against gram positive bacteria. Attempts to extend the antimicrobial activity of penicillin G had led to the development of semi-synthetic ampicillin in the year 1961 which is effective against gram positive and gram negative organisms (William and Petri, 2011). Ampicillin is effectively being employed against major clinical pathogenic organisms that are involved in equine infections (Meijer *et al.,* 2000). Ampicillin is active against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus viridans* and *Streptococcus pneumoniae*, *Gonococci*, *Neisseria meningitidis* and *Hemophilus influenzae* (Greenwood, 2008). Plasma protein binding plays vital role in antimicrobial therapy as it influences pharmacokinetic and pharmacodynamic activity of a drug. Binding of penicillin with plasma proteins enhances their biological half-life through prevention of β-lactam ring hydrolysis as well as decreased renal elimination (Hornish and Kotarski, 2002). Protein bound fraction of the drug does not have any antibacterial activity; it is only unbound drug that possesses this activity (Craig and Kunin, 1976) and so, for evaluation of the potential efficacy of antibacterial, its plasma protein binding should be known.

Several methods have been published for the determination of ampicillin in pure form or pharmaceutical formulations as well as in biological samples included by spectrophotometric methods (Askal *et al.,* 1991; Sastry *et al.,* 1998) HPLC method (Burns *et al.,* 1991) post-

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column degradation method (Haginaka *et al.,* 1987). The spectrophotometric method selected in the present study was that originally described by Smith *et al.* (1967) for determination of ampicillin, based on copper facilitated formation of stable acid degradation product which shows maximum absorption at 320 nm. This paper describes the suitability of this assay for determination of ampicillin in pharmaceutical formulations as well as in plasma samples by UV-Visible spectrophotometer.

MATERIALS AND METHODS

Reagents

Analytical grade copper sulphate, citric acid, disodium hydrogen phosphate, potassium-dihydrogen phosphate were purchased from Merck (Germany). Analytical standard of ampicillin, dialysis tubing cellulose membrane was procured from Sigma Aldrich Co, USA.

***Copper sulphate solution*:** Copper sulphate pentahydrate of 3.93 g was dissolved in 1 litre water.

***Buffer solution* pH 5.2:** 0.1 M citric acid solution of 464 ml was mixed with 0.2 M disodium hydrogen phosphate solution of 536 ml. Adjust the pH, if necessary, to 5.2±0.05 with the citric acid or disodium hydrogen phosphate solution. To 15 ml of the copper sulphate solution add the buffer at pH 5.2 to a volume of 1 litre. (1 ml of this solution contains 15 µg of copper).

Experimental animals

The study was conducted on clinically presented horses of age group 8-12 years of age with 385-480 kg body weights. Animals were monitored during the collection of blood samples. The protocol of the study was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (Order No. F.No.25/4/2019-CPCSEA, dt: 20/03/2019).

Collection of blood samples

Blood samples were collected into heparinised vials from jugular veins. Plasma was separated by centrifugation at 3000 rpm for 15 minutes and stored at -20°C till analysis further.

In vitro plasma protein binding

In vitro binding of ampicillin to plasma proteins was determined by employing the equilibrium dialysis technique (Gupta *et al*., 2006; Singh *et al.,* 2019). The dialyzing bags (4 Å pore size), 10 cm long were washed in running tap water and soaked overnight in phosphate buffer. Ampicillin concentrations (3.125, 6.25, 12.5, 25, 50, 100 µg/ml) were prepared in pooled plasma separated from blood taken from apparently healthy horses. Each dialyzing bag was knotted at one end before filling 5ml of plasma containing known amount of drug and the other end was then securely tied. Each bag was immersed in separate tubes containing 5 ml of phosphate buffer (0.2 M; pH 7.4; disodium hydrogen phosphate 11.3 g, potassium-dihydrogen phosphate 2.7 g, added to 1000 ml of distilled water) and the tubes were incubated at 37°C for 24 h with intermittent shaking. At the end of incubation period phosphate buffers as well as contents of the dialyzing bags were separately analysed for the concentration of ampicillin by spectrophotometric assay in triplicate. The extent of *in vitro* plasma protein binding of ampicillin was calculated by the following equation.

$$\text{Percent of drug bound to plasma protein} = \frac{CP' - CB}{CP} \times 100$$

where,

CP' = Concentration of drug in plasma after incubation

CB = Concentration of drug in buffer after incubation

CP = Concentration of drug in plasma before incubation

Binding capacity of the plasma protein to ampicillin (b_1) and the dissociation rate constant of protein drug complex (K_b) were calculated by the method of Pilloud (1973).

Apparatus

Ultraviolet-Visible-Spectrophotometer (Eppendorf™, USA) with 1 cm matched quartz cells was used for all spectral measurements. pH meter (Thermo scientific® Digital pH Meter Orion star A111), electronic weighing balance (A&D Instruments, India Pvt. Ltd.), refrigerated centrifuge (Eppendorf™, USA), hot water bath incubator shaker (Narang Scientific Works Pvt. Ltd), thoroughly clean and dried glasswares were used in the present study such as measuring cylinders, volumetric flasks and beakers

were procured from Borosil Glass Works Ltd. (India). The glass wares were washed with hot water and detergent, rinsed with distilled water and finally dried in an oven prior to use.

Spectrophotometric method for ampicillin detection

Preparation of standards

Stock solution of 100 µg/ml ampicillin was prepared in distilled water. The standard solutions were prepared by dilution of stock solution with distilled water in a concentration range of 0.5, 1, 2, 4, 6, 8 and 10 µg/ml. The stock solution containing glass vials were wrapped in aluminium foil and stored under deep refrigeration (-20ºC) until use.

Calibration curve for ampicillin standards

Buffered standard solutions of ampicillin at a range of concentrations 0.5, 1, 2, 4, 6, 8 and 10 µg/ml were assayed by the specified procedure.

Spectrophotometric assay

A simple and specific spectrophotometric method given by Smith *et al.* (1967) was applied for the determination of ampicillin at 320 nm, based on acid degradation product of penicillin at 75°C in the presence of citrate buffer (pH 5.2) and traces of copper salt. In the study, it was observed that this method has limitation, and the detection of ampicillin not beyond 1.0 µg/ml. We have now extended the method to the assay of ampicillin in horse plasma for *in-vitro* plasma protein binding assay.

Method validation

The method was validated for linearity, precision (inter-day and intra-day), selectivity and sensitivity according to the ICH guidelines. Linearity could be established with standard plots constructed for ampicillin in the range of 1-10 µg.ml^{-1} (Table 1). The experiment was repeated thrice on the same day and additionally on two consecutive days to determine intra-day and inter-day precision, respectively. Calibration curve was plotted between ampicillin concentrations against absorbance readings as shown in Fig. 1.

Plasma sample extraction procedure

Plasma (0.2 ml) from heparinized blood is taken in a 1.5 ml centrifuge tube containing absolute ethanol (0.4 ml). After mixing in vortex for few seconds and centrifuging for 5 min at 4500 RPM, clear supernatant (0.2 ml) was taken into a 5 ml tube with citrate buffer solution (0.8 ml; pH 5.2) containing copper (15 µg/ml). The tube was agitated at 120 strokes/min, incubated at 75 °C for 30 min and then cooled in ice. The sample is taken in a 1.5 ml cuvette and read at 320 nm wavelength against the blank given by the residual non-incubated fraction of the supernatant.

Table 1: Linearity data for ampicillin spiked in horse plasma (n= 3)

Drug	Range	Regression parameters	
		Regression equation	R^2 value
Ampicillin	(1-8 µg.ml^{-1})	y = 0.1519x + 0.0287	0.991

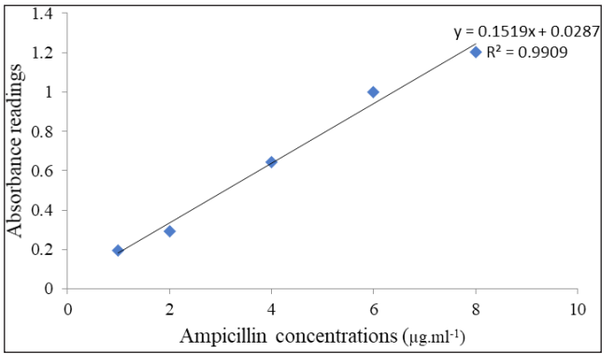

Fig. 1: Calibration curve between ampicillin concentrations against absorbance readings at wavelength 320 nm wavelength

RESULTS AND DISCUSSION

In the present study specific UV-Visible spectrophotometric method described by Smith *et al.* (1967) was used for the determination of ampicillin, based on the copper facilitated formation of the stable acid degradation products, for which the presence of the intact antibiotic molecule is essential. The method was found to be simple, accurate, economical and reproducible. The drug concentrations were found to be linear in the range of 1-8 µg/ml and the correlation coefficient value of 0.991 indicates that method was linear.

Table 2: *In vitro* plasma protein binding and kinetic constant of ampicillin in apparently healthy horses

Ampicillin concentration (µg.ml^{-1})							
	Protein binding (%)						
Experiment No.	**3.12**	**6.25**	**12.5**	**25**	**50**	**β_i**	**K_β**
1	12.8	12.2	14.4	12.0	12.6	0.32×10^{-6}	0.0031×10^{-9}
2	12.2	12.4	13.6	11.4	13.8	0.38×10^{-6}	0.0034×10^{-9}
3	13.4	12.8	13.0	11.2	14.8	0.31×10^{-6}	0.0041×10^{-9}
Mean ± SEM	12.8 ± 0.3	12.4 ± 0.3	13.6 ± 0.6	11.5 ± 0.4	13.7 ± 0.6	$0.34 \times 10^{-6} \pm 0.02 \times 10^{-6}$	$0.003 \times 10^{-9} \pm 0.0003 \times 10^{-9}$

Over all protein binding (%) Mean ± SEM is 12.8 ± 0.07 %

β_i = Association rate constant (mol/kg) and K_β= Dissociation rate constant (mol); $\beta_i = 0.34 \times 10^{-6} \pm 0.02 \times 10^{-6}$ mol.kg^{-1}; $K_\beta = 0.003 \times 10^{-9} \pm 0.0003 \times 10^{-9}$ mol.

In-vitro plasma protein binding of ampicillin in the present study was carried out in apparently healthy clinical horses cases by equilibrium dialysis technique. *In vitro* plasma protein binding and kinetic constants are given in Table 2. The protein bound portion of an antibacterial drug does not have any antimicrobial activity and only free drug in the body possesses pharmacodynamic activity. The extent of protein binding can also directly influence the therapeutic efficacy of antibacterial drug (Craig and Kunin, 1976). The unbound fraction of drug is also desired for distribution and clearance (Wise *et al.,*1980 and Yamada *et al.,* 1981). The efficacy of β-lactam antibiotics has direct correlation with the time length for which its unbound concentration remains above minimum inhibitory concentration as percent of dosing interval (Andes and Craig, 2002). So for evaluation of the potential efficacy of ampicillin its plasma proteins binding should be known in horses.

It was reported that ampicillin is slightly plasma protein bound drug (<25%) (Ziv and Sulman, 1972). The extent of protein binding percent for ampicillin obtained in the present study was 12.8±0.07%. However, Durr *et al.* (1976) reported that ampicillin is very slightly bound to proteins in horses (6.8-8 %) assayed by equilibrium dialysis technique. (β_i) binding capacity of drug to plasma proteins and (K_β) dissociation rate constant of protein drug complex quantitatively describe the drug protein interaction. In the present study (β_i) and (K_β) of ampicillin were $0.34 \times 10^{-6} \pm 0.02 \times 10^{-6}$ mole.g^{-1} and $0.003 \times 10^{-9} \pm 0.0003 \times 10^{-9}$ mole, respectively. The higher value of β_i than K_β indicated that binding of ampicillin to plasma proteins was relatively faster than dissociation of protein drug complex in horses. The plasma samples and buffer were also quantified for the drug levels by using HPLC, and the results were in agreement with the spectrophotometric method.

CONCLUSION

The advantage of UV-Vis Spectrophotometer application is its quick analysis ability, cost effective and easy to use. The procedure described can be used for routine assay of samples for ampicillin covering a wide potency range. Low plasma protein binding aspect of ampicillin obtained in the present study indicates that as an unbound drug it can easily diffuse into the extra cellular fluid through the capillary pores and attain an almost equal concentration to that in plasma; which can influence its antimicrobial efficacy.

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