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Short communication

Determination of non-protein bound phenylbutazone in bovine plasma using ultrafiltration and liquid chromatography with ultraviolet detection

E.J. Ian De Veau[1]

U.S. Food and Drug Administration, Center for Veterinary Medicine, 8401 Muirkirk Road, Laurel, MD 20708, USA

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Abstract

A liquid chromatographic procedure using UV detection was coupled with ultrafiltration for the quantitation of free phenylbutazone in bovine plasma, in the range of 20 ng/ml to 2.0 μg/ml. Whole plasma samples (0.5 to 1 ml) were placed in a 2-ml centrifugal concentrator with a molecular-mass cut-off membrane of 10 000 and centrifuged at 4500 *g* for 2 h at 4°C using a fixed angle rotor. The ultrafiltrate was transferred to an LC vial with a 200-μl insert and 100 μl was injected into an LC system. The chromatographic system used a C_{18} reversed-phase column connected to a UV detector set at 264 nm. The mobile phase was 0.2 *M* sodium phosphate buffer (pH 7)–methanol (1:1). Recoveries of phenylbutazone from protein-free plasma water fortified at levels of 20 ng/ml to 2 μg/ml ranged from 91 to 93%, with relative standard deviations (R.S.D.s) ranging from 1 to 4%. The concentration of incurred non-protein bound phenylbutazone obtained from a cow intravenously dosed twice with 2 g phenylbutazone, 8 h apart, was 111, 26 and 11 ng/ml for 2, 72 and 104 h post first phenylbutazone dose, respectively.

Keywords: Protein binding; Phenylbutazone

1. Introduction

Many drugs bind reversibly to plasma proteins. Such bindings can have significant pharmacodynamic and pharmacokinetic influences, and the concentration of the non-protein bound fraction of the drug can offer explanations for the behavior of that drug within the body [1]. Drug–protein binding impacts apparent distribution and elimination half-lives, rates of metabolic transformation, volume of distribution, and clearance. Drug–protein binding will also influence the amount of free bioactive drug circulating throughout the body and the diffusion of the drug into tissues and pharmacological receptor sites to cause a physiological effect. For drugs having narrow therapeutic indices, the monitoring of the non-protein bound portion is critical to prevent drug concentrations from becoming toxic.

Phenylbutazone (4-butyl-1,2-diphenyl-3,5-pyrazolidinedione) is a non-steroidal anti-inflammatory drug approved by the U.S. Food and Drug Adminis-

[1]Tel.: +1-301-827-8158, Fax: +1-301-827-8170; e-mail: edeveau@bangate.fda.gov

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tration (FDA) for use in dogs and horses [2]. There is evidence of its extra-label use in food producing animals for the treatment of various inflammatory conditions, including mastitis in lactating dairy cows [3]. This raises food safety concerns, because phenylbutazone can cause several adverse reactions in sensitive humans. The more serious ones include aplastic anemia, agranulocytosis, and various gastrointestinal disorders, such as gastrointestinal bleeding [4].

The elimination half-life of phenylbutazone varies from species to species [5]. This variation is thought to be primarily due to differences in metabolic rates of biotransformation between species [3]. However, the degree of protein binding may have a significant role in some species. For example, in one study with non-lactating cows, the mean elimination half-life was reported to be about 36 h with more than 98% of plasma phenylbutazone bound to protein [3]. By comparison, the goat, another ruminant species, has an elimination half-life of about 15 h with 60% of plasma phenylbutazone bound to protein [6]. However, a more recent publication has called into question this relatively large amount of unbound phenylbutazone in the goat [7].

Recently, several methods have been published for simplified and rapid extraction and analysis of total plasma phenylbutazone [8–10]. Though there are various methods for the determination of free plasma phenylbutazone [3,7,11], they all have the disadvantage of having relatively long sample preparation time. For example, in the equilibrium dialysis method, sample preparation requires about 24 h [11]. Conversely, ultrafiltration can be used to quickly prepare plasma water samples to determine the amount of free drug [12]. Recently, ultrafiltration has been used for the determination of non-protein bound phenylbutazone in goat plasma [7]. However, the authors used a long extraction procedure for phenylbutazone after ultrafiltration which limits the number of samples that can be analyzed within a day, and they did not test the ultrafiltration membrane for phenylbutazone binding. A simpler method using ultrafiltration coupled with reversed-phase liquid chromatography (LC) and UV detection [13] for the rapid determination of free phenylbutazone in bovine plasma is described in the current publication.

2. Experimental

2.1. Collection and preparation of plasma samples and phenylbutazone administration

Control blood samples were taken from a lactating cow from the jugular vein using heparinized syringes. Blood samples were centrifuged at 1900 *g* for 15 min, and the resulting supernatant plasma was transferred to polypropylene tubes. Plasma samples were stored at 4°C if used within 16 h or at <-60°C for later use. For the generation of incurred plasma phenylbutazone, a cow was given two intravenous doses, 8 h apart, via the jugular vein. Each dose contained 4.4 mg phenylbutazone/kg body mass. After phenylbutazone dosing, serial venous blood samples (10–20 ml) were collected from the contralateral jugular vein and plasma samples were prepared as above. Plasma samples prepared from blood collected at 2, 72 and 104 h post first phenylbutazone dose were analyzed to determine the concentration of non-protein bound phenylbutazone.

2.2. Ultrafiltration of bovine plasma

Bovine plasma samples were ultrafiltered by placing 0.5 to 1 ml of plasma into a 2-ml centrifugal concentrator with a molecular-mass cut-off membrane of 10 000 (Centricon-10, Amicon, Beverly, MA, USA). The samples were centrifuged at 4500 *g* for 2 h at 4°C using a fixed angle rotor. Aliquots of the ultrafiltrates were transferred to individual LC sample vials with 200-μl inserts. At this point samples are ready for injection onto the LC system for analysis.

2.3. Preparation of phenylbutazone stock, calibration and fortification solutions

Phenylbutazone stock solution was prepared by weighing 10 mg of phenylbutazone (Sigma, St. Louis, MO, USA) and dissolving in 100 ml of 0.02 *M* Na-phosphate buffer, pH 7–methanol (1:1). This solution is stable for 30 days when stored at 4°C. To prepare calibrations standards in the range of 10 to 500 ng/ml, 0.5 ml of the stock solution was diluted to 100 ml with 0.02 *M* Na-phosphate buffer, pH

7–methanol (1:1) to prepare a working standard (phenylbutazone concentration=500 ng/ml). Calibration standards at phenylbutazone concentrations lower than 500 ng/ml were prepared by dilution of the working standard with 0.02 *M* Na-phosphate buffer, pH 7–methanol (1:1).

To prepare a high phenylbutazone concentration working standard for the generation of calibration standards greater than 500 ng/ml, 1 ml of phenylbutazone stock solution was diluted to 10.0 ml with 0.02 *M* Na-phosphate buffer, pH 7–methanol (1:1). Calibration standards in the range of 0.5 to 2.0 μg/ml were prepared by further dilution with 0.02 *M* Na-phosphate buffer, pH 7–methanol (1:1). Calibration standards were prepared fresh for each set of analyzes.

A low fortification solution was prepared by diluting 0.5 ml of the phenylbutazone stock solution with 0.02 *M* Na-phosphate buffer, pH 7–methanol (1:1) to 50 ml. A high fortification solution was prepared by diluting 10 ml of phenylbutazone stock solution to 50.0 ml with 0.02 *M* Na-phosphate buffer, pH 7–methanol (1:1). The use of methanol in the fortification solutions could not be avoided due to limited solubility of phenylbutazone in aqueous solutions.

2.4. Determination of amount of phenylbutazone binding to the ultrafiltration membrane

To determine if phenylbutazone binds to the ultrafiltration membrane, milliliter quantities of control protein-free bovine plasma water were prepared using preparatory centrifugal ultrafilters (Centriprep-10, Amicon) according to the manufacturer's instructions. The protein-free plasma water prepared this way was ultrafiltered again using the Centricon-10 as above to make sure that all proteins above 10 000 had been removed. This protein-free plasma water was then fortified. To obtain 20 and 100 ng phenylbutazone/ml fortified plasma water, 60.0 and 300.0 μl of the low fortification solution were added to 6.0 ml of protein-free plasma water, respectively. To obtain 1 or 2 μg phenylbutazone/ml fortified protein-free plasma water, 150.0 or 300.0 μl of the high fortification solution were added to the 6.0 ml of protein-free plasma water, respectively. After fortification, the plasma water was vortexed and an aliquot was analyzed to determine actual phenylbutazone concentration. The remaining volume of fortified plasma water was divided into 0.5 ml portions and transferred into individual Centricon-10 ultrafilters. The plasma water was then re-centrifuged. The ultrafiltrate was collected, transferred to an LC vial, and chromatographically analyzed as described below.

2.5. Chromatographic analysis

The concentration of phenylbutazone in protein-free plasma water was determined by injecting 100 μl protein-free plasma water onto a C_{18} reversed-phase analytical column (Ultracarb 5 ODS (20) 150×4.6 mm; Phenomenex, Torrance, CA, USA) preceded by a C_{18} guard column (Supelcosil LC-18DB, 20×4.6 mm; Supelco, Bellefonte, PA, USA). Column temperature was maintained at 35±0.1°C using an LC column oven. The mobile phase was 0.02 *M* Na-phosphate buffer, pH 7–methanol (1:1) with a flow-rate of 1.0 ml/min. Phenylbutazone was detected at a wavelength of 264 nm. To achieve greater precision, a low phenylbutazone calibration curve was generated using phenylbutazone standards in the range of 10 to 500 ng/ml for test analyte concentrations of less than 500 ng/ml. For test analyte concentration of greater than 500 ng/ml, a high phenylbutazone calibration curve was generated using standards in the range of 0.5 to 2.0 μg/ml. Test analyte concentrations were determined from the non-weighted linear regression equations established between peak height and concentration of calibration standards.

3. Results and discussion

Fig. 1 illustrates a typical chromatogram for control protein-free plasma water and protein-free plasma water containing biologically incurred phenylbutazone at a measured concentration of 25.4 ng/ml. Retention time of phenylbutazone was in the range of 8 to 11 min, depending upon the lot number of the C_{18} column. Small changes in the amount of methanol in the mobile phase can be made if

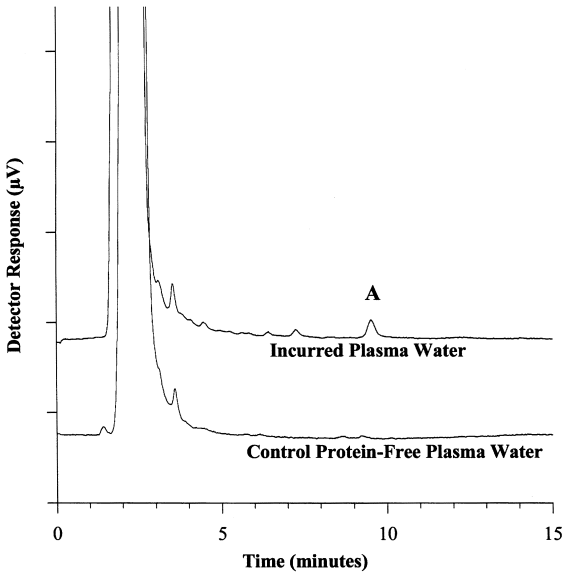

Fig. 1. Composite chromatogram of control protein-free plasma water and biologically incurred phenylbutazone in protein-free plasma. Incurred protein-free plasma water was measured as having 25.4 ng phenylbutazone/ml. Phenylbutazone is identified as peak A.

phenylbutazone does not elute within the above time frame. The retention time of phenylbutazone will start to shift after repeated daily use of the column due to column fouling. Flushing the column periodically with 100% methanol was found to restore the retention time of phenylbutazone back to its original value and to extend the life of the column. No significant, endogenous protein-free plasma water component elutes within 6 min of phenylbutazone. The limit of detection (S/N =3.0) and limit of quantitation (S/N=5.5) [14] were determined to be 3.4 and 6.2 ng/ml, respectively. All calibration curves were linear with r^2 values of at least 0.999. Table 1 lists the recoveries for control and fortified protein-free plasma water extracts at the levels of 20.0 ng/ml to 2.0 μg/ml. Results for incurred phenylbutazone residues in protein-free plasma water for selected time points from the single cow are listed in Table 2.

The above method for preparation of protein-free plasma water for non-protein bound plasma phenylbutazone analysis is simple and straightforward. Coupled to an LC system with an autosampler and an automatic data acquisition system, more than 60 plasma samples can be analyzed in a day. The ease of this procedure permits a more complete monitoring of the concentration and pharmacokinetics of phenylbutazone in the protein-free plasma water fraction using relatively small plasma sample volumes. The performance of the method was not validated for the active metabolite of phenylbutazone, oxyphenbutazone. However, the determination of free oxyphenbutazone concentration in plasma was not considered important, since the plasma concentration of total oxyphenbutazone in cattle never rises above 1% of the parent [3,11,15,16]. Therefore, the concentration of free oxyphenbutazone in cattle is most likely minimal, and it should have little to no effect in cattle either pharmacologically or toxicologically.

Table 1
Recoveries of phenylbutazone from control and fortified protein-free plasma water

Phenylbutazone fortification level	*n*	$\bar{x}$% Recovered	R.S.D. (%)
0 (control)	5	Not detected	–
20 ng/ml	13	89.9±5.8	6.42
100 ng/ml	13	93.7±2.8	3.03
1 μg/ml	10	93.1±0.7	0.78
2 μg/ml	10	93.4±1.7	1.85

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Table 2
Analyzed levels for non-protein bound phenylbutazone in plasma water

Time post first dose (h)	*n*	$\bar{x}$ Amount recovered (ng/ml)
2	3	110.9±4.2
72	3	26.4±1.1
104	3	11.1±0.6

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