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Note

High-performance liquid chromatographic method for determination of phenylbutazone in bovine milk with special reference to the fat content in milk

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From the aspect of food hygiene drug monitoring in products from food producing animals is very important, as residue levels might be a potential hazard to the consumer. In Sweden, the nonsteroidal antiinflammatory agent phenylbutazone (PBZ) is used for treating cows suffering from arthritis and laminitis, although knowledge of PBZ residue levels in milk is lacking. Methods for determination of PBZ in different body fluids are reported [1—6], but not in milk from lactating dairy cows. Attempts have been made to study PBZ concentrations in human milk [7, 8] by a method which is well documented for detection of PBZ in plasma or serum [1]. However, this method is inexpedient for determinations of PBZ in cows' milk, due to matrix problems and poor sensitivity. We here describe a method which allows the determination of minute amounts of PBZ in cows' milk by a reversed-phase high-performance liquid chromatographic (HPLC) technique and UV detection.

EXPERIMENTAL

Chemicals

PBZ was obtained form Lääke/Farmos Yhtymä Oy (Åbo, Finland). All other chemicals used were purchased from E. Merck (Darmstadt, G.F.R.). Diethyl ether and ethanol were of spectrographic grade, light petroleum, *n*-hexane and methanol of p.a. grade and ammonia was of suprapure grade.

Apparatus

The HPLC system consisted of a Constametric III pump, a SpectroMonitor

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III variable-wavelength UV detector (LDC, Riviera Beach, FL, U.S.A.) and a Rheodyne 7120 injector (Rheodyne, Berkeley, CA, U.S.A.) with a 100-μl loop. The output signal (0.005 a.u.f.s.) was integrated and calculated by a chromatograph Control Module II (LDC) and plotted on an LDC two-channel thermal printer plotter. A Shimadzu UV-210 A spectrophotometer was used to measure the UV spectra of PBZ.

Chromatographic technique

The column (150 × 4.0 mm I.D.) was packed with LiChrosorb RP-18, 5-μm particle size (E. Merck) and the pre-column (70 × 2.0 mm I.D.) with CO:Pell ODSC-18, diameter 30—38 μm (Whatman, Clifton, NY, U.S.A.).

The mobile phase was prepared from 550 ml of a 0.02 *M* phosphate buffer, pH 7.0, and 450 ml methanol. The flow-rate was set at 1.200 ml/min. Detection of PBZ was achieved at 264 nm. With the system operating at room temperature, the retention time of PBZ was 6 min.

To protect the columns and to keep the retention time constant the system was washed with 50 ml methanol after every 50 injections.

Extraction procedure

To 1 ml of milk were added 1.1 ml of ethanol (99.5%)—ammonia (25%) (10:1) and 2.4 ml of diethyl ether. The tubes were vigorously shaken for 1 min and left for 5 min. Thereafter 2.4 ml of light petroleum (b.p. 40—60°C) were added. The tubes were turned upside down ten times and left for 1 h. The ether phase was carefully sucked off and discarded. To the remaining aqueous phase were added 5 ml of *n*-hexane and 0.4 ml 3 *M* hydrochloric acid. The tubes were shaken for 30 min and centrifuged for 30 min. The organic phase was transferred to a new tube and then evaporated to dryness in a nitrogen stream. The residue was redissolved in 0.5 ml of mobile phase. Of this an aliquot of 100 μl was injected onto the column.

Calibration curve

The calibration curve for determination of PBZ by HPLC was prepared by dissolving PBZ in methanol to a concentration of 0.1 mg/ml. Appropriate amounts of this standard solution were added to milk samples to yield concentrations of 0.1, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3, 1.5 μg/ml. These samples were then treated as described above. The peak areas were plotted versus the concentrations. The calibration curve was linear ($y = 0.977x + 0.011$). The correlation coefficient was 0.9987. From the slope of the calibration curve a reference factor was calculated which was used for automatic calculation of the concentrations of PBZ in the milk samples.

Distribution of PBZ between the fat and aqueous fractions in milk

From milk, spiked with 1 μg/ml PBZ, four whole milk samples were taken. The remaining milk was centrifuged in order to separate the fats from the aqueous fraction and four samples were collected from the aqueous fraction. The concentrations were compared in these two sets of samples.

Determination of PBZ in milk with different fat concentrations

PBZ to a concentration of 1 μg/ml was added to milk from six cows, with fat concentrations ranging from 3.5—4.4%, to Swedish standard milk (3%) and to Swedish low fat milk (0.5%). The PBZ concentrations were determined in the different milk samples and the correlation coefficient between the PBZ concentrations and the fat concentrations was calculated.

RESULTS AND DISCUSSION

As milk is a suspension of fat droplets in an aqueous phase, a drug may be unevenly distributed in the two phases depending on its physiological properties. The distribution of a drug between a complicated fat fraction and a water phase which also contains proteins, makes analysis of drugs in milk a difficult task. The high fat content in milk may interfere with the sampling, the extraction procedure and the HPLC separation. PBZ, which is a lipid-soluble weak acid (pK_a = 4.5), was found to be distributed both in the fat and in the water fraction. In our study, the concentration of PBZ in the water fraction was half the concentration found in the whole milk. Providing there is a fat content of 4% in the milk the concentration of PBZ is 25 times higher in the fat fraction. This means that to have representative samples the milk must be carefully mixed before collection of samples. Moreover, it is known that the fat concentration in milk increases during the course of milking [9] so the foremilk may contain less PBZ than the milk at the end of milking. To collect representative samples for quantitative calculations of PBZ, the udder must be emptied and the obtained milk carefully mixed before sampling.

The methods used by others [7, 8] for detection of PBZ in human milk starts with acidifying a 1-ml sample. When we used this application on cows' milk we found that acidification sometimes led to a gel formation which disturbed further extraction of PBZ. We also found that repeated injections onto the HPLC column of extracted but not fat-reduced samples increased the pressure drop across the columns and decreased the retention times, the separation efficiency and the peak height, probably due to accumulation of milk components on the columns. Similar effects are reported by Wiese et al. [10] after repeated injections of plasma samples only treated with methanol for precipitation of the serum proteins. To minimize the negative effects on the extraction procedure and to improve separation efficiency, the milk fats were reduced according to the initial steps of the international standard method for fat determination in milk [11]. The fats were dissolved and extracted to an ether phase under alkaline conditions leaving PBZ, which is ionised at high pH, in the aqueous phase. This procedure eliminated the problems with the gel formation. However, the defatting step was not sufficient to completely eliminate the components deteriorating the HPLC columns but the negative effects were almost negligible. However, to avoid an extensive pressure drop across the columns, it is recommended that the pre-column should be changed or the system washed after about 50 injections. The decrease in separation efficiency produced during 50 injections was not a problem as the peak area was constant. This was verified by measuring the peak area after injections of standard solutions. If the peak height instead of the area

is used for quantitation, the problems may be more pronounced, even if an internal standard is used, since the decreases in peak heights for substances with different retention times are not necessarily comparable — a fact which we have seen in our laboratory when simultaneously determining chloramphenicol and its monsuccinate in plasma from pigs by an HPLC technique [12]. Such disturbance phenomena must be considered when the internal standard is chosen.

Since the fat content in cows' milk varies among breeds, individuals, nutrition and lactation stages, it is important that an assay monitoring drugs in milk is independent of the fat content. When tested in the method described above, we could not find any correlation (r = 0.016) between the fat content and the measured PBZ concentrations in the milk. Thus, no corrections had to be made for different fat concentrations in the milk samples.

In recent publications [4—6] UV detection of PBZ has been performed at acidic pH and 240 or 254 nm. As can be seen in Fig. 1, PBZ has different absorption maxima at different pH values. The dashed line is the spectrum at pH 7.0 with a maximum at 264 nm ($\epsilon = 2.2 \cdot 10^4$ l mol^{-1} cm^{-1}) and the solid line is the spectrum at pH 3.0 with a maximum at 237 nm ($\epsilon = 1.3 \cdot 10^4$ l mol^{-1} cm^{-1}). This means that the sensitivity is increased if UV detection is performed at neutral pH and 264 nm compared to pH 3.0 and 240 nm.

By assaying ten in vitro samples containing 100 ng/ml PBZ, the recovery was calculated and found to be 89% with a coefficient of variation of 5.7%. A PBZ concentration in milk as low as 20 ng/ml could be readily detected.

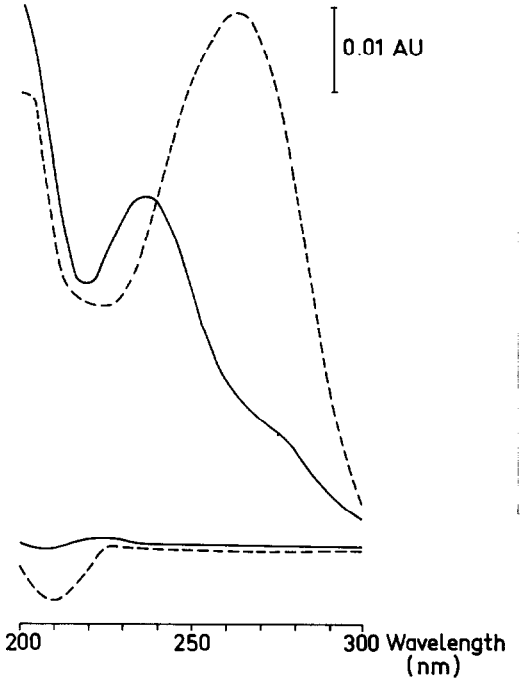

Fig. 1. UV spectra at different pH values for PBZ (1 μg/ml) dissolved in phosphate buffer—methanol (55:45). The horizontal lines show the UV spectra of the solvents. (- - -) pH = 7.0, (—) pH = 3.0.

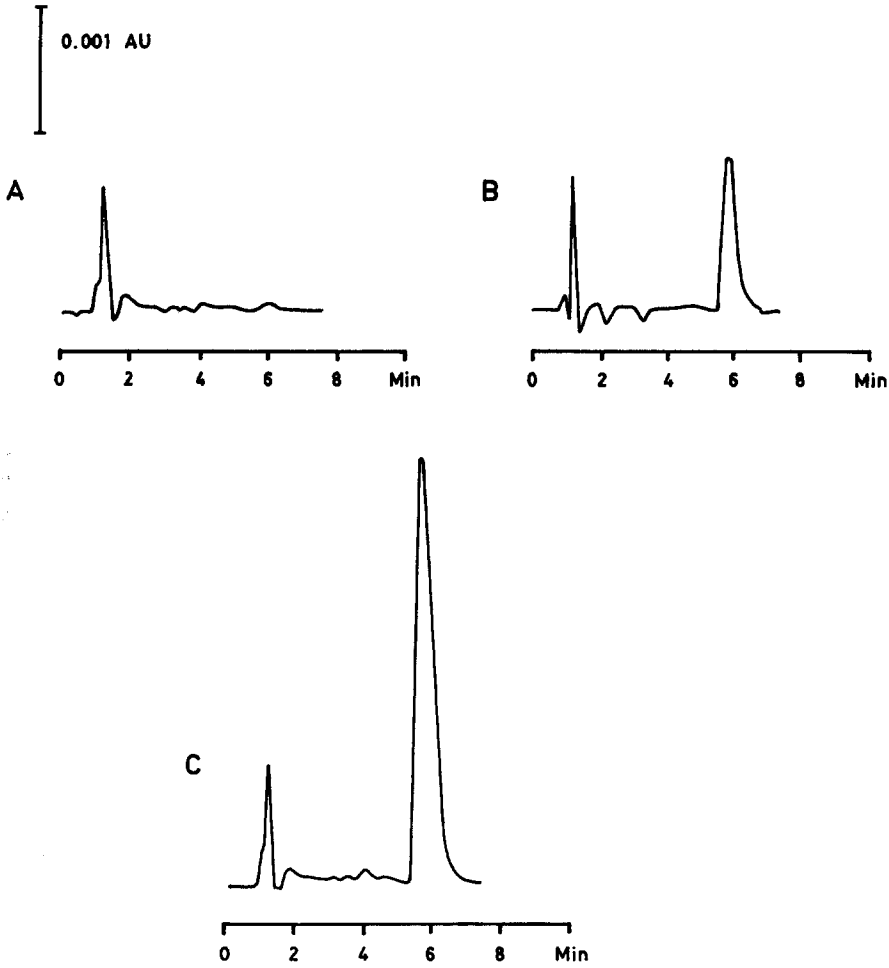

Fig. 2. Chromatograms of milk extract: (A) from blank milk; (B) from milk spiked with PBZ (50 ng/ml); (C) from milk of a cow after administration of 2.5 g of PBZ 16 and 24 h before sampling.

Fig. 2 shows chromatograms obtained by this method. Fig. 2A illustrates milk without PBZ, Fig. 2B milk to which was added 50 ng/ml PBZ and Fig. 2C milk from a cow after administration of 2.5 g of PBZ 16 and 24 h before sampling.

In summary, a method for quantitative determinations of PBZ in cows' milk is described. The extraction procedure used should be generally applicable as a first step in determinations of lipid-soluble weak acids in milk. The method is independent of the fat concentration of the milk samples and permits estimation of minute amounts of PBZ as the HPLC conditions are optimised for highest sensitivity.

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