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Note

Determination of orotate in ruminant milk by high-performance liquid chromatography

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Orotate is an intermediate in pyrimidine biosynthesis and therefore a component of all cells. The concentration of orotate in the tissues, including bovine tissues, is low [1]. Significant concentrations of orotate were found in milk of animals in all three main families of the suborder Ruminantia [2].

Until now, orotate has been determined either by microbiological assays [3], chemical colour reactions [4, 5], or enzymatically [6]. However, differences between the results of several authors are mainly due to assay procedure and to a lesser extent to real differences [3]. Also, because these methods are either expensive or time-consuming, we decided to develop a rapid high-performance liquid chromatographic (HPLC) method to screen large numbers of bovine milk samples for orotate, to study the effects of diet on orotate concentration in milk of ruminants. Orotate was shown to induce fatty liver in rats, and cow's milk is the major source of orotate in the human diet. Knowledge of the influence of the cow's diet on orotate excretion in milk may provide a means for regulating orotate in milk if evidence suggests that orotate in milk poses a problem [7].

EXPERIMENTAL

Reagents

Orotic acid monohydrate (6-carboxy-2,4-dihydropyrimidine) was obtained from Sigma (St. Louis, MO, U.S.A.).

Water was filtered through the Milli-Q Purification System (Millipore, Bedford, MA, U.S.A.). All other reagents were of analytical reagent grade. Mobile phase was degassed and filtered using Pyrex filter holders with 0.5-μm pore diameter filters from Millipore.

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Apparatus

The HPLC system used consisted of a Waters M6000A liquid chromatographic pump (Waters Assoc., Etten-Leur, The Netherlands), a Waters M441 UV/VIS discrete absorbance detector, a Waters automatic sample injector (WISP 710B) and a Spectra Physics SP4100 computing integrator (Spectra Physics, San Jose, CA, U.S.A.).

The column was a reversed-phase μBondapak C_{18} (10 μm; 30 cm × 3.9 mm I.D.) from Waters Assoc., combined with a guard column (2.0 cm × 4.5 mm I.D.) packed with 40-μm C_{18}/Corasil (Waters Assoc.).

Procedure

Samples of milk were obtained from Friesian dairy cows during the morning milking. Milk was deproteinized with trichloroacetic acid (TCA) according to ref. 1, with some modifications: 1 ml of milk was diluted with 4.0 ml of chilled water and 0.2 ml of 50% (w/v) TCA was added; after at least 4 h standing at 4° C, the sample was mixed thoroughly and centrifuged (15 min, 4000 *g*). After filtering the samples, orotate was determined by HPLC.

The chromatographic procedure was as follows. The mobile phase consisted of 0.05 *M* phosphate, pH 7.0. Flow-rate was 1.5 ml/min. A 30-μl aliquot of the filtered supernatant was injected into the column. Column effluent was monitored at 280 nm and at 0.2 absorbance units full scale. Peak areas were calculated into concentrations (mg/l) by the integrator.

RESULTS AND DISCUSSION

The standard calibration curve for orotate in 2% (w/v) TCA was linear in the concentration range 0—202 mg/l. The regression line was (triplicate measurements): $Y = 1053.7X - 64.6$ in which Y = peak area and X = orotate concentration. The standard deviation of the slope was 4.6 and the correlation coefficient (R) was 0.9999.

The chromatographic pattern of orotate standard (Fig. 1A) and of orotate in the filtered supernatant of milk (Fig. 1B) are shown in Fig. 1.

The accuracy of the determination of orotate by HPLC was measured with addition of standards: instead of 4.0 ml of water, 3.0 ml of water and 1.0 ml of orotate standard at four different levels were added to milk in duplicate. After deproteinization with TCA, the orotate concentration was determined and the recovery was calculated (Table I). The average recovery was 100.13 ± 1.50% (± standard deviation, S.D.).

To measure the precision of the determination of orotate, we deproteinized 18 milk samples in triplicate and the concentration in each filtered supernatant was measured in duplicate. The average coefficient of variation (C.V.) of the concentration of orotate due to variations in the chromatographic system (pump, injector, detector, integrator) was 0.55 ± 0.43% (± S.D., $n = 54$). The average coefficient of variation of the total determination (triplicate measurements) was 1.29 ± 0.81% (± S.D., $n = 18$). The concentration of these milk samples ranged from 9.5 to 119.1 mg/l.

The day-to-day variation of the determination of orotate was 0.92% at 62.6 mg/l and 0.57% at 125.5 mg/l orotate in milk ($n = 10$).

TABLE I

RECOVERY OF OROTATE ADDED TO COW'S MILK

Orotate conc. in milk (mg/l)	Orotate added (mg/l)	Measured conc. (mg/l)	Recovery (%)
64.9	60	124.7	99.8
64.9	60	128.6	103.0
64.9	120	183.2	99.1
64.9	120	188.0	101.7
55.3	30	85.5	100.2
55.3	30	84.8	99.4
55.3	90	143.1	98.5
55.3	90	144.3	99.3

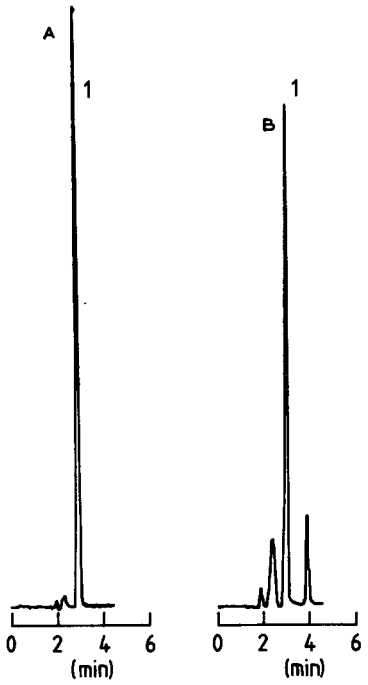

Fig. 1. Chromatographic profiles of (A) 67.25 mg/l standard orotate (1), and (B) orotate (1) in the filtered supernatant of milk (58.7 mg/l).

We did not find any interfering peaks in the 2250 milk samples analysed up to now.

The coefficients of variation of the determination of orotate by chemical analysis and by bioassay with *Lactobacillus jugurt* were 6.9% and 12.8%, respectively [3]. Therefore, the determination of orotate in milk by HPLC is rapid, sensitive and precise. The automated HPLC system is very useful in determining orotate in large numbers of milk samples (more than 1000).

Preliminary results show that the mean concentration of orotate in the milk of 2250 cows is 53.8 mg/l. The distribution was asymmetrical with skewness to the right. The average standard deviation of the distribution was 21.1 mg/l. These results agree with the results obtained by Jesse et al. [8].

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