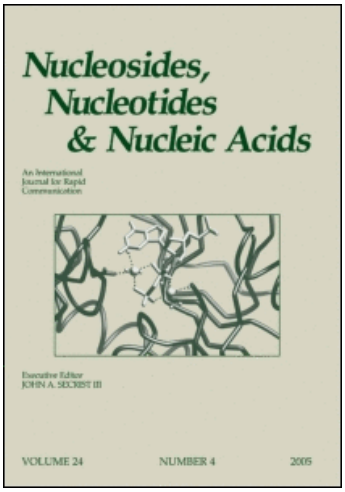

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Quantitative Analysis of Orotic Acid in Urine by RP-HPLC

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QUANTITATIVE ANALYSIS OF OROTIC ACID IN URINE BY RP-HPLC

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Abstract: we describe a new RP-HPLC assay to measure orotate concentration in urine. The method is rapid, sensitive and precise. It is particularly suitable for the diagnosis of inherited metabolic diseases and deranged orotate metabolism.

Orotate is an intermediate in pyrimidine biosynthesis and its quantitative determination is of diagnostic value in some inherited metabolic diseases (1). Many methods have been developed to measure orotate concentration in biological samples (2,3,4,5,6,7,8). They are rather cumbersome, time-consuming, most of them lack sensitivity, specificity or resolution or involve costly equipment. We developed a new RP-HPLC assay in determining orotate in urine. It is rapid, easy to perform, gives good sensitivity and reproducibility.

A 0.5 ml urine sample, filtered through a 0.22 µm Millex-GS filter were forced through a Sep-Pak C18 cartridge (Waters) activated with 2 ml of acetonitrile-water (60:40, v/v) and 5 ml of water. The cartridge was washed twice with 0.5 ml of water. The eluates were pooled and acidified to pH 1.5-2.0 with 37% HCl. A 25 µl sample was chromatographed isocratically with 3.2 mM HCl at flow rate of 1 ml/min on two coupled columns LiChrospher 100, RP-18 125x4 mm (ID), 5 µm particle size (Merck). Column effluent was monitored at 280 nm . The elution was performed at room temperature.

The chromatographic profiles of a sample of pooled human urine and of the same sample with added orotic acid are shown in Fig.1. Peak purity was verified by spectral analysis (Fig.2) and by mass spectrometry. Following the above

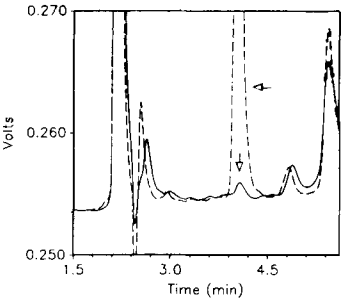

FIG.1 chromatograms of:
— urine sample control
– – urine sample control with added orotate solution (10µg/ml)
◁— orotate peak

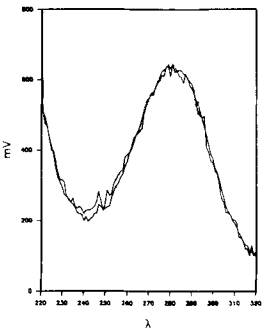

FIG.2 spectral analysis of orotate: upslope and downslope of the peak

procedure, mean orotate retention time is 4.06±0.022 min (mean ± SD ; n=10).

The calibration curve was obtained by processing aliquots of aqueous orotate solutions at different concentrations (from 0.1 to 100 µg/ml) (Fig.3). The regression equation is Y=68.1X + 3.6 in which X=orotate concentration in the diluited sample (µg/ml), Y=peak area/1000. The standard error of the slope is 0.169. The 95% confidence limits are 68.476 and 67.648, and the correlation coefficient is 0.9999. The detection limit is <0.1 µg/ml. The recovery of orotate, added to a pooled urine sample, was 99.72±3.36 (mean±SD) in the concentration range of 0.2-50 µg/ml.

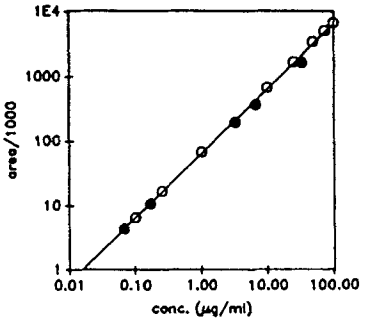

FIG.3 Ocalibration curve with aqueous orotate solutions
● recovery of orotate added to urine sample

Our method is sensitive and precise. The HPLC procedure is rapid (15 min from injection to injection) and sample preparation is quick and easy. These characteristics make our method suitable for the determination of urinary excretion of orotate in a control population and for the diagnosis of inherited metabolic diseases and deranged orotate metabolism.

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