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Journal of Chromatography B, 660 (1994) 176–179

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Determination of orotic acid in serum by high-performance liquid chromatography

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First received 7 September 1993; revised manuscript received 17 May 1994

Abstract

A simple method for the determination of orotic acid in serum is described. The analyses were carried out using a strong anion-exchange column. Orotic acid was separated isocratically with 0.8 *M* formic acid (pH 2.8, adjusted with 10 *M* NaOH)–methanol (65:35, v/v) at a flow-rate of 1.0 ml/min. Absorbance at 275 nm was recorded for quantification. Validation yielded a detection limit of 0.07 μg/ml and a quantification limit of 0.25 μg/ml. The maximum within-day and day-to-day coefficients of variation were less than 6%. The method has the advantage that samples can be easily prepared.

1. Introduction

Orotic acid is a precursor of the biosynthesis of pyrimidine nucleotides. The cerebroprotective and cardioprotective effects that may result can be demonstrated in animal experiments. Such effects have not been verified hitherto in healthy volunteers or patients [1].

Clinical studies require that orotic acid be determined in biological fluids. For years, it has been common practice to determine orotic acid spectrophotometrically [2]. However, interferences with endogenous substances occur that can be avoided only by laborious sample preparation and by measuring blanks [3,4].

Another method uses blanks obtained by enzymatic conversion of orotic acid to a non-absorbing compound and subsequent differential measurements [5]. HPLC methods for the determination of orotic acid have described, including separation on reversed-phase [6] or anion-exchange columns [7]. A column-switching HPLC method using a reversed-phase column for sample clean-up and a cation-exchange column for separation was described for urinary orotic acid [8]. In this work, we developed a simple and rapid method of preparing serum samples and a chromatographic separation modified according to Brusilow and Hauser [7].

2. Experimental

2.1. Equipment

The following equipment was used: an HPP5001 HPLC pump from Laboratórní Přístroje (Prague, Czech Republic), an SPD-6A spectrophotometer from Shimadzu (Kyoto,

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SSDI 0378-4347(94)00264-6

Japan) and a Nucleosil 100-10SB 10-μm analytical column (250 × 4 mm I.D.) from Macherey–Nagel (Düren, Germany). The injection valve used was an HPLC six-port valve from Knauer (Bad Homburg, Germany).

2.2. Chemicals and reagents

Formic acid (85%) was purchased from Laborchemie (Apolda, Germany), sodium hydroxide from Chemapol (Prague, Czech Republic), methanol from Merck (Darmstadt, Germany), trichloroacetic acid from Reanal (Budapest, Hungary), orotic acid from Wörwag (Stuttgart, Germany) and 2-nitrobenzoic acid used as an internal standard (I.S.), from Riedel-de Haën (Seelze, Germany).

2.3. Calibration

For calibration, 10-ml aliquots of human serum were mixed with 50 μl of a stock standard

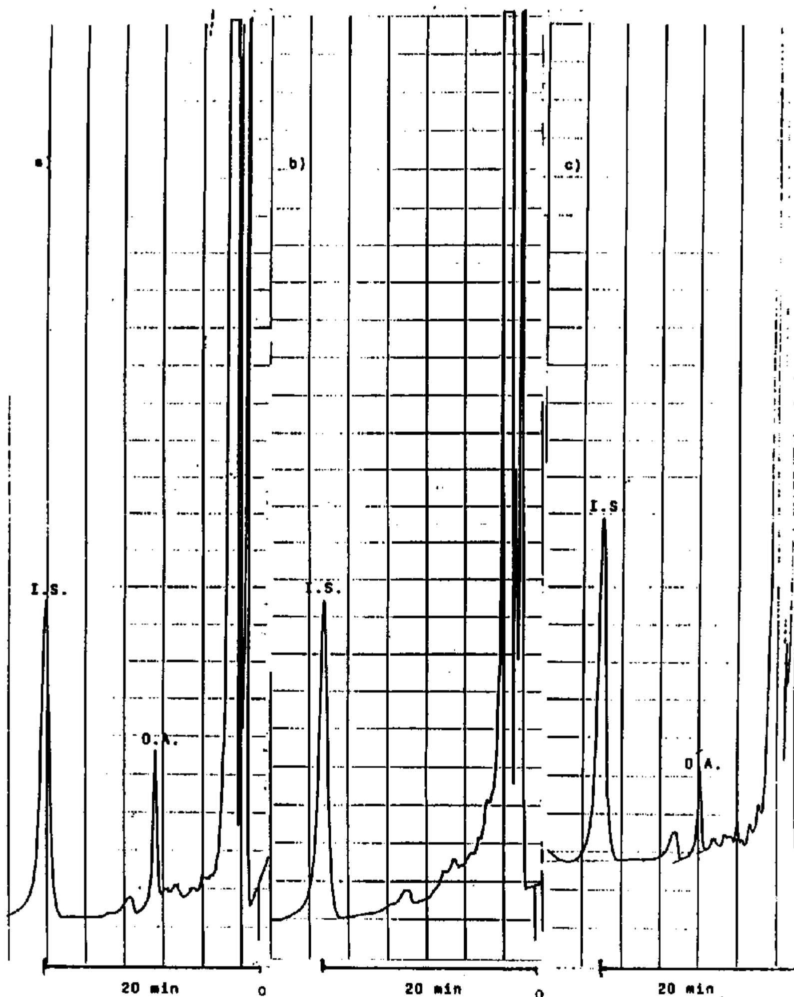

Fig. 1. Chromatograms of (a) serum spiked with internal standard (I.S.) and 2 μg/ml of orotic acid (O.A.), (b) blank and (c) a patient's sample containing 0.6 μg/ml of orotic acid.

Table 1
Calibration data, limit of detection (LOD) and limit of quantification (LOQ)

Range[a] (μg/ml)	Intercept	Slope	r	LOD (μg/ml)	LOQ (μg/ml)
0.5–10	0.0399	0.2362	0.9994		
0.25–2.5	0.0079	0.2242	0.9993	0.07	0.25

[a] $n = 10$.

solution of 20 mg of orotic acid in 100 ml of water. 2-Nitrobenzoic acid (100 mg) in 100 ml of water was used as the I.S. solution.

2.4. Sample preparation

A 10-μl volume of I.S. solution and 200 μl of trichloroacetic acid (10%, w/v) were added to 500 μl of serum. After mixing for 10 s on the Whirlimixer and centrifugation for 10 min at 2600 *g*, the clear supernatants were transferred into a glass tube.

2.5. Chromatographic conditions

A mixture of 0.8 *M* formic acid (pH 2.8, adjusted with 10 *M* NaOH) and methanol (65:35, v/v) at a flow-rate of 1.0 ml/min at room temperature was used as the mobile phase for isocratic separation. The detection wavelength was 275 nm and the injection volume was 20 μl.

3. Results and discussion

3.1. Chromatography

We were unable to obtain the separation on a reversed-phase column. Brusilow and Hauser [7] presented a method for measuring urinary orotic acid on a strong anion-exchange column and we adapted this method for determination of orotic acid in serum.

Serum blanks from seventeen healthy volunteers were tested and did not reveal any interferences from endogenous substances.

Typical chromatograms are shown in Fig. 1.

3.2. Validation

The results of calibration are given in Table 1. Adequate linearity was achieved in the range tested and the calibration functions had good regression parameters. The limits of detection and quantification were derived from multiple measurements in the low concentration range. The values found were extrapolated to the point where, with a confidence of 95%, the detector signal was three times higher than the signal from a blank sample. This concentration value is defined as the limit of detection (LOD). Multiplication of the LOD by 3.5 yields the limit of quantification, where the results have an uncertainty of 33%. The resulting limits were found to be sufficient for pharmacokinetic studies.

The results of the precision studies are given in

Table 2
Within-day and day-to-day precision ($n = 10$)

Concentration added (μg/ml)	Maximum found (μg/ml)	Minimum found (μg/ml)	Mean (μg/ml)	S.D. (μg/ml)	R.S.D. (%)
Within-day					
0.5	0.55	0.44	0.50	0.03	5.6
10.0	10.19	9.94	10.01	0.14	1.9
Day-to-day					
5.0	5.66	4.79	5.34	0.24	4.5

Table 2. The assay results are stable over a period of ca. ten weeks.

In conclusion, an HPLC method for the determination of orotic acid in human serum has been developed. Sample preparation is easy and rapid. The validation parameters permit orotic acid in serum to be determined in the course of pharmacokinetic studies.

Acknowledgement

The technical assistance of Mrs. Dorothea Baehs is gratefully acknowledged.

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