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Short Communication

Improved high-performance liquid chromatographic assay for the determination of ethionamide in serum

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ABSTRACT

A solid-phase extraction (SPE) method was developed to simplify the preparation of human serum prior to high-performance liquid chromatography of ethionamide (ETA). Octadecyl SPE columns were used. Serum constituents were removed from the column with water, and ETA was eluted with methanol. Samples were evaporated to dryness, reconstituted in mobile phase, and assayed. The method is reproducible, with a recovery of ETA of 64%, comparable to the more tedious liquid–liquid extraction method for ETA.

INTRODUCTION

Ethionamide (ETA, 2-ethylthioisonicotinamide) is a second-line agent with activity against various mycobacteria, including *M. tuberculosis, M. leprae,* and *M. avium-intracellulare* (MAI) [1,2]. A number of assay methods for the measurement of ethionamide in serum have been developed [3–6]. The method of Shepard *et al.* [5] has proven to be particularly useful because of its sensitivity, specificity, and reproducibility. We describe a solid-phase extraction (SPE) method which simplifies the preparation of serum for assay.

EXPERIMENTAL

Chemicals

Ethionamide and prothionamide (PTA, internal standard) were donated by

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Medimpex Northamerica (New York, NY, U.S.A.). Stock solutions (200 μg/ml) were prepared fresh daily by dissolving the drugs in 0.02 *M* disodium phosphate buffer–acetonitrile (50:50, v/v).

Extraction procedure

Aliquots (200 μl) of serum were pipetted into 100 mm × 13 mm borosilicate tubes. The PTA internal standard stock solution (4 μl) was added to each sample. Samples were vortex-mixed for 5 s.

Octadecyl SPE columns (1 ml) (J. T. Baker, Phillipsburg, NJ, U.S.A.) were prepared with one column volume of methanol followed by one column volume of purified water (Milli-Q system, Millipore, Milford, MA, U.S.A.). The 204-μl samples were pipetted onto the columns and drawn onto the matrix using low vacuum (2 mmHg). The columns were washed with 400 μl of purified water to remove plasma constituents. Excess water was removed with a 50-μl rinse of 10:90 (v/v) water–methanol, followed by drying under vacuum (15 mmHg) for 15 min. The collection rack holding 1.8-ml polypropylene collection vials was then placed into the vacuum manifold. ETA and PTA were extracted with 1000 μl of methanol using low vacuum (2 mmHg). After all of the methanol was drawn onto the matrix, the pressure was increased to 10 mmHg for 10 s. The collection vials were withdrawn from the vacuum manifold, placed in a water bath (40°C), and dried under nitrogen (approximately 15 min).

The samples were reconstituted with 200 μl of 0.02 *M* disodium phosphate buffer–acetonitrile (75:25) and vortex-mixed for 5 s. The entire contents of each collection vial was transferred to an autosampler vial prior to analysis.

Liquid chromatography

Analyses were performed using a Waters (Milford, MA, U.S.A.) Model M6000A pump, a Spectra-Physics (San Jose, CA, U.S.A.) Model 8875 autosampler, a Waters 440 ultraviolet detector set at 254 nm, 0.01 a.u.f.s., and a Rainin (Woburn, MA, U.S.A.) Dynamax® HPLC Method Manager Data Package installed on a Macintosh Plus computer (Apple Computers, Cupertino, CA, U.S.A.). A reversed-phase system was used consisting of an Alltech (Deerfield, IL, U.S.A.) 5-μm Hypersil ODS cartridge column (250 mm × 4.6 mm I.D.) preceded by an Alltech 5-μm Hypersil ODS cartridge guard (10 mm × 4.6 mm I.D.) column. The mobile phase was 0.02 *M* disodium phosphate buffer–acetonitrile (75:25), which was filtered (0.45 μm), degassed by sonication, and delivered at a flow-rate of 1.5 ml/min (0.138 kPa). Injections of 20 μl were made, with a run time of 12 min per sample.

Standard curves

Standard curves were constructed to encompass the anticipated range of serum concentrations found in patients taking ethionamide. Blank serum was spiked with ethionamide to give concentrations of 0.2, 0.5, 1.0, 5.0, 10.0, and 20.0

μg/ml. Aliquots (200 μl) of each standard were then prepared using the above extraction procedure. The line of best fit was determined using weighted linear regression analysis of the ETA/PTA peak-height ratio (y) *versus* the theoretical concentration of ETA (x).

Selectivity

The selectivity of the method with respect to all other antimycobacterial agents available in the United States, as well as common agents such as aspirin and acetaminophen, was evaluated. Aqueous solutions containing 20–75 μg/ml of the above drugs were injected into the analytical system. [Rifampin was dissolved in water–acetonitrile (50:50).]

RESULTS

Analytical procedure

A representative chromatogram of the extract of a serum sample obtained 2 h after ingestion of 500 mg ethionamide is shown in Fig. 1. The retention times of ETA and PTA are 4.28 and 7.24 min, respectively. Separation of ETA and PTA was complete, with a resolution factor (R_s) of 5.93.

Concentrations of ETA were determined using the peak-height ratio of ETA to PTA. Linear regression analysis using a weight of $1/y^2$ provided the best fit over the 2 log concentration range. The three-day validation produced slopes ranging from 0.426 to 0.486 and intercepts ranging from 0.002 to 0.041. None of the intercepts differed significantly from zero. The response of the detector was linear over the 2 log range of concentrations tested, with a practical limit of detection of 0.2 μg/ml ETA (peak height-to-noise ratio $\geq$ 5:1). Overall recovery of ETA, calculated by comparing peak heights of extracted serum samples with those of directly injected water samples, was 64%. Analysis of the data for overall

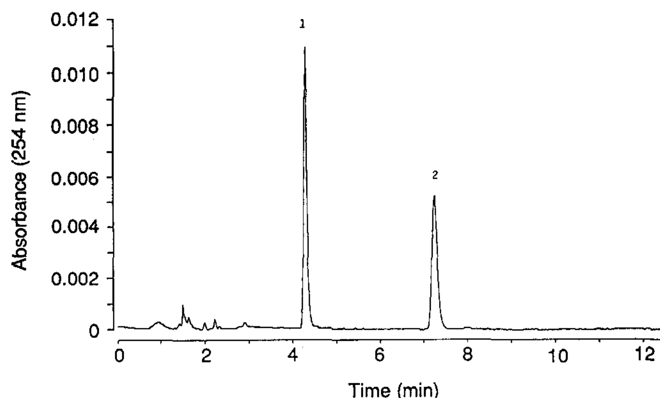

Fig. 1. Chromatogram of an extract of serum from a patient 2 h after the ingestion of 500 mg ETA. Peaks: 1 = ETA; 2 = PTA (internal standard).

assay precision produced a coefficient of variation of 4.91% (range 1.02% at 20 μg/ml to 5.79% at 0.2 μg/ml).

The following drugs did not coelute with either ETA or PTA: acetaminophen, aspirin, amikacin, capreomycin, ethambutol, isoniazid, kanamycin, *p*-aminosalicyclic acid, pyrazinamide, streptomycin, and rifampin. Phenobarbital was shown to coelute with ETA. Concomitant treatment with phenobarbital should be avoided when measuring ETA serum concentrations.

CONCLUSION

The HPLC method described for the determination of ethionamide in serum is specific for ETA, unlike bioassays which measure ETA and the active sulfoxide metabolite. The method shows sensitivity, specificity, and recovery similar to that described by Jenner and Ellard [3] and by Shepard *et al.* [5], while replacing the liquid–liquid extraction with a simple, reproducible solid-phase extraction method.

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