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Methods for the estimation of pyrazinamide and pyrazinoic acid in body fluids

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Methods for the estimation of pyrazinamide in serum and of pyrazinamide and pyrazinoic acid in urine using an anion-exchange resin (Dowex-1) have been described. Recoveries were quantitative, and the sensitivity was 5 µg/ml for ail 3 estimations. In serum, pyrazinoic acid at a concentration of 50 µg/ml did not interfere with the estimation of pyrazinamide. In urine, pyrazinamide and pyrazinoic acid could be separated from each other and estimated even when the 2 compounds were present together at concentrations of 2000 µg/ml each.

Studies on the pharmacology of pyrazinamide have gained importance in view of the suitability of this drug for short-course chemotherapy of tuberculosis[1]. Pyrazinamide is usually estimated in body fluids by reaction with alkaline nitroprusside. However, pyrazinoic acid– the principal metabolite of pyrazinamide, also reacts with nitroprusside under these conditions. For separation of pyrazinamide from pyrazinoic acid in body fluids, Ellard[2] used a solvent extraction procedure while Subbammal and associates[3] used an anion-exchange resin. In the latter method, described from our Centre for the estimation of pyrazinamide in serum, the entire eluate is used, and if a repetition becomes necessary, the entire procedure starting from serum has to be repeated. To overcome this difficulty, we have modified the procedure slightly and have also extended it to the estimation of pyrazinamide and pyrazinoic acid in urine.

Material and Methods

Estimation of pyrazinamide in serum : To 3.5 ml of serum was added 1.8 ml of 10 per cent trichloroacetic acid and the contents were stirred and centrifuged. Three ml of the protein-free supernate was applied to a column (75 x 6 mm) of Dowex 1- x 8 (Cl-form, 200-400 mesh) and the column was washed with small amounts of water till 10 ml of the eluate was collected. Three ml of this eluate was treated with 0.5 ml of a freshly prepared solution of 0.2 per cent sodium nitroprusside in water followed by 0.5 ml of 2N sodium hydroxide solution. The tubes were left at room temperature for 15 min and the optical densities were recorded at 495 nm in one cm cells in a Unicam SP 600 spectrophotometer.

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Estimation of pyrazinamide and pyrazinoic acid in urine : Three ml of urine were applied to the column of the anion-exchange resin and pyrazinamide was eluted from the column by washing it with small amounts of water till 15 ml of the eluate was collected. For pyrazinoic acid, the column was first washed with 15 ml of water followed by small amounts of 0.5M sodium chloride solution till 15 ml of the eluate was collected. Three ml of the respective eluates were treated with alkaline nitroprusside and the optical densities recorded as described for serum.

Results

Recovery of pyrazinamide from serum : On each of 5 occasions, pyrazinamide was set up, in duplicate, in concentrations of 0, 2.5, 5, 10, 25, 50 and 100 µg/ml in horse serum. The serum samples were randomised and the optical densities were recorded as described before. Another set of pyrazinamide concentrations (as above), in duplicate, in horse serum was processed directly, *i.e.,* 3 ml of the protein-free supernate (after a 3 in 10 dilution) was directly reacted with alkaline nitroprusside solution and the optical densities recorded. Water standards in the concentrations mentioned were also set up concurrently on each occasion and processed directly. The geometric mean optical densities are presented in Table I.

Results showed that with the ion-exchange method, the optical density was proportional to the concentration of pyrazinamide over the range tested. Comparison with serum and water standards processed directly showed that recovery of pyrazinamide from serum was quantitative. The coefficient of variation for replicate estimations with the ion-exchange method was 19.5 per cent for a concentration of 2.5 µg/ml and 3-5 per cent for concentrations of 5-100 µg/ml.

Readings obtained with water blanks were much higher than those obtained with horse serum, the mean optical densities being 0.137 and 0.044, respectively.

Blank sera from different patients : Blood was collected from 15 patients not receiving pyrazinamide, serum separated and the serum samples processed for estimation of pyrazinamide. The blank readings for the different specimens ranged from 0.090 to 0.098, but the differences were not significant statistically ($P>0.2$). The mean optical density for the 15 patients was 0.094, which was considerably lower than the mean optical density with water standards (0.137) and considerably higher than that with horse serum (0.044). This suggests that it is essential to employ human serum blanks for estimating serum pyrazinamide concentrations in human subjects. As the blank readings from different patients were similar, pooled human serum can be employed for setting up blanks.

Recovery of pyrazinamide and pyrazinoic acid from urine : On each of 5 occasions, pyrazinamide and pyrazinoic acid were set up, in duplicate, in concentrations of 0, 5, 10, 25, 50 and 100 µg/ml in pooled normal urine (from volunteers). Optical densities were determined by the ion-exchange method described above as well as by the direct processing method.

The geometric mean optical densities are presented in Table II. The results indicate that with both pyrazinamide and pyrazinoic acid, Beer's law is followed

Table I. Optical density readings with pyrazinamide in horse serum and water

	Method	Mean optical density* with the following concentrations of pyrazinamide (µg/ml)						Mean optical density with blanks
		2.5	5	10	25	50	100	
Horse serum	Ion-exchange	0.020	0.036	0.064	0.139	0.284	0.558	0.046
	Direct	0.020	0.038	0.068	0.142	0.288	0.555	0.044
Water	Direct	0.015	0.033	0.062	0.141	0.286	0.562	0.137

*After subtracting values obtained with blanks.

Table II. Optical density readings with pyrazinamide and pyrazinoic acid in urine

	Method	Mean optical density* with the following concentrations of pyrazinamide and pyrazinoic acid (µg/ml)					Mean optical density with blanks
		5	10	25	50	100	
Pyrazinamide	Ion-exchange	0.024	0.047	0.111	0.222	0.441	0.015
	Direct	0.023	0.045	0.108	0.223	0.439	0.033
Pyrazinoic acid	Ion-exchange	0.022	0.044	0.107	0.210	0.413	0.019
	Direct	0.022	0.044	0.105	0.211	0.413	0.031

*After subtracting values obtained with blanks.

over the range 5-100 µg/ml. Comparison with urine standards processed directly indicates a quantitative recovery of both the compounds from urine. With the ion-exchange method, the coefficient of variation for replicate estimations at a concentration of 5 µg/ml was 5.7 per cent for pyrazinamide and 4.8 per cent for pyrazinoic acid, and 3-6 per cent for both in concentrations of 10-100 µg/ml.

Sensitivity of the methods : Sensitivity of the method has been regarded as the lowest concentration where the coefficient of variation for replicate estimations is 10 per cent or less. In the estimation of pyrazinamide in serum, the coefficient of variation was 19.5 per cent at a concentration of 2.5 µg/ml and 4.8 per cent at 5 µg/ml; in urine, the coefficients of variation at 5 µg/ml were 5.7 per cent for pyrazinamide and 4.8 per cent for pyrazinoic acid. The sensitivity of all the three methods is, therefore, 5 µg/ml.

The sensitivity of the method for estimation of pyrazinamide in serum

(5 µg/ml) was not a serious limitation as this concentration is well below the minimum inhibitory concentration of pyrazinamide, reported to be between 9 and 16 µg/ml. in a Tween-free high citrate medium (pH 5.6)[4,5].

Interference studies

Pyrazinamide in serum : On each of 3 occasions, pyrazinoic acid in concentrations of 0 and 50 µg/ml was added to pyrazinamide concentrations of 0, 10, 25 and 50 µg/ml set up in horse serum, and the optical densities determined after randomisation of the specimens. The blank readings for pyrazinamide were the same both in the absence and in the presence of pyrazinoic acid *(viz.,* 0.087). The mean optical densities were 0.060, 0.142 and 0.274 with pyrazinamide concentrations of 10, 25 and 50 µg/ml in the absence of pyrazinoic acid, and 0.070, 0.149 and 0.274 respectively, in the presence of pyrazinoic acid 50 µg/ml, demonstrating that pyrazinoic acid did not interfere with the estimation of pyrazinamide. However, if a 30 per cent solution of trichloroacetic acid is used, instead of 10 per cent solution employed here, exchange of pyrazinoic acid is diminished considerably (unpublished observation) as the stronger trichloroacetic acid is exchanged in preference to pyrazinoic acid and the latter is eluted out with water itself.

Pyrazinamide and pyrazinoic acid in urine : On each of 4 occasions, pyrazinoic acid 0 and 2000 µg/ml was added to pyrazinamide concentrations of 0 and 2000 µg/ml in pooled normal urine and the optical densities determined for both the compounds after a 3 in 50 dilution of the respective eluates. Concurrently, pyrazinamide and pyrazinoic acid standards of 2000 µg/ml in urine were processed directly after a 3 in 50 dilution of the samples. The mean optical densities for pyrazinamide 2000 µg/ml alone and in the presence of pyrazinoic acid 2000 µg/ml were 0.592 and 0.590, respectively. On direct reaction, pyrazinamide 2000 µg/ml gave a reading of 0.593. The mean optical densities for pyrazinoic acid 2000 µg/ml alone and in the presence of pyrazinamide 2000 µg/ml were 0.562 and 0.561, respectively. On direct reaction, pyrazinoic acid 2000 µg/ml gave a reading of 0.555. These findings demonstrate that in urine, effective separation of pyrazinamide from pyrazinoic acid was achieved even when these two compounds were present together at concentrations of 2000 µg/ml each, and also that the elution of both was quantitative even at these high concentrations.

Interference due to some antituberculosis drugs : Streptomycin, p-aminosalicylic acid, thioacetazone, rifampicin, ethambutol, ethionamide and cycloserine in concentrations up to 500 µg/ml did not interfere with the estimation of either pyrazinamide or pyrazinoic acid (0, 10 and 25 µg/ml) in urine. Neither did isoniazid, but acetylisoniazid caused a significant over-estimation of pyrazinamide. For example, acetylisoniazid 100 µg/ml gave an optical density reading equivalent to that obtained with pyrazinamide 7.5 µg/ml; the estimation of pyrazinoic acid was, however, not affected. It is, therefore, necessary to withhold isoniazid for at least 48 h prior to any pharmacological investigations with pyrazinamide.

Discussion

The methods described here for the estimation of pyrazinamide and pyrazi-

noic acid are fairly simple and quite specific, as prior separation of the two is achieved with the use of an anion-exchange resin. When an aqueous solution containing a mixture of pyrazinamide and pyrazinoic acid is passed through an anion-exchange resin, pyrazinoic acid is retained on the column and is eluted only with a neutral salt solution (0.5 M sodium chloride solution was used in our studies).

This procedure can also be used to estimate pyrazinoic acid in serum. Pyrazinoic acid can be eluted from the column with 10 ml of 0.5 M sodium chloride solution following removal of pyrazinamide with water. The method will have only a limited applicability as the sensitivity is likely to be 5 µg/ml, and Ellard[2] has shown that peak serum concentrations of pyrazinoic acid did not exceed 10 µg/ml in African patients receiving pyrazinamide 3 g.

In the estimation of pyrazinamide and pyrazinoic acid reported here, water blanks gave higher readings than horse serum, human serum or urine blanks. (The lower readings obtained with horse and human sera and urine may be due to an inhibition of the nitroprusside reaction due to increasing concentrations of ammonia present in these samples[2]). Dilution of the samples (or the eluates) with water would therefore result in higher optical density readings. When such a dilution becomes necessary (for samples containing high concentrations of pyrazinamide or pyrazinoic acid), it is essential to dilute the blanks also similarly.

In the estimation of pyrazinamide and pyrazinoic acid in urine, it has been observed that a column containing the anion-exchange resin can be re-used at least 10 times (unpublished observation) as the resin is regenerated at the time of elution of pyrazinoic acid from the column with 0.5 *M* sodium chloride solution. In the case of the estimation of pyrazinamide in serum, however, it will be necessary to regenerate the resin by washing it with 0.5 *M* sodium chloride solution following elution of pyrazinamide with water. In routine practice, it is advisable to wash the column with 15 to 20 ml of water, after treatment with sodium chloride solution, prior to application of the fresh sample.

The solvent extraction procedure described by Ellard[2] for estimation of pyrazinamide in serum is more sensitive (1 µg/ml) than that reported here. The two methods are of the same sensitivity for estimation of pyrazinamide and pyrazinoic acid in urine. In the procedure described by Ellard[2], a slight interference of 3.2 per cent due to pyrazinamide in the estimation of pyrazinoic acid has been reported; also, care has to be taken to avoid prolonged contact of pyrazinamide with alkali. The methods described by us here have fewer steps. Further, pyrazinamide and pyrazinoic acid do not interfere with the estimation of each other in urine; nor does pyrazinoic acid interfere with the estimation of pyrazinamide in serum.

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References

1. Fox, W. and Mitchison, D.A. Short-course chemotherapy for pulmonary tuberculosis. *Am Rev Respir Dis* **111** (1975) 325.
2. Ellard, G.A. Absorption, metabolism and excretion of pyrazinamide in man. *Tubercle* **50** (1969) 144.
3. Subbammal, S., Krishnamurthy, D.V., Tripathy, S.P. and Venkataraman, P. Concentrations of pyraxinamide attained in serum with different doses of the drug. *Bull WHO* **39** (1968) 771.
4. Dickinson, J.M. and Mitchison, D.A. Observations *in vitro* on the suitability of pyrazinamide for intermittent chemotherapy of tuberculosis. *Tubercle* **51** (1970) 389.
5. Carrada Bravo, T., Ellard, G.A. and Mitchison, D.A. Reappraisal of the activity of morphozinamide against *M. tuberculosis.* *Tubercle* **56** (1975) 211.

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