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Note

High-performance liquid chromatographic analysis of nitrate in urine and feces

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Nitrate is important to human health because of methemoglobin formation in infants [1] and because carcinogenic N-nitroso compounds can form in vivo, either in the stomach [2] or in an infected urinary bladder [3], after ingestion of nitrate and the corresponding amines. Several methods, which have been described for the analysis of nitrate, are either subject to interferences by other compounds present in the samples or they are time-consuming because of lengthy clean-up steps [4,5] and/or conversion to nitro compounds [6], nitrite [7,8], or ammonia [9]. Reduction to nitrite requires subsequent diazotization [4] or additional chemical reactions [10,11]. furthermore, cadmium used for nitrate reduction and azo dyes that are subsequently produced are themselves toxic [12,13]. Two direct high-performance liquid chromatographic (HPLC) methods for nitrate and nitrite have been reported, by Thayer and Huffacker [14] for plant extracts, and by Gerritse [15] for sewage. In preliminary experiments, we found that the former [14] did not provide clean separations from other compounds present in urine and feces, while the inferior peak resolution is obtained in the latter [15]. We therefore set out to find conditions for assay of nitrate in complex biological samples.

MATERIALS AND METHODS

Reagents

Analytical-grade reagents and degassed, glass distilled, deionized water (specific resistance 16—18 MΩ/cm) were used.

Biological samples

Urine and feces from Sprague-Dawley rats or humans (authors) were pre-

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pared as previously described [16]. A nitrate-free 0.5-μm or 0.2-μm filter (Millipore, Bedford, MA, U.S.A.) was used before HPLC analysis.

High-performance liquid chromatography

The following equipment was used: a Perkin-Elmer Series 2 pump (Perkin-Elmer, Norwalk, CT, U.S.A.); a Whatman Partisil 10 SAX (Whatman, Clifton, NY, U.S.A.) or Ultrasil AX (Altex, Berkley, CA, U.S.A.) 25-cm anion-exchange column; a 6-cm guard column packed with Whatman HC Pellosil or Whatman Partisil 10 SAX; a Rheodyne 7010 sample injector (Rheodyne, Cotati, CA, U.S.A.); and a Gilson Holochrome HM/HPLC spectrophotometer (Gilson Medical Electronics, Middleton, WI, U.S.A.). After digitization, the output was interfaced to a Microflop Descope computer (Charles River Data Systems, Natick, MA, U.S.A.).

Columns were routinely eluted with 22.5 m*M* aqueous phosphate buffer, pH 2.35. For a given type of sample the pH was altered to a value where nitrate eluted alone.

Reduction of nitrate to ammonia

Sample (5 ml of urine or intestinal contents), 5 ml of 0.4 *N* sodium hydroxide and 1 g of Devarda's Alloy (J.T. Baker, Phillipsburg, NJ, U.S.A.) were combined and heated at 100°C for 15 min. Phosphoric acid (0.1 ml) was then added and the precipitate was removed by centrifugation at 10,000 *g*.

Reduction of nitrate to nitrite

Samples were passed through a column (10 × 0.5 cm) of coarse mesh cadmium (E. Merck, Darmstadt, G.F.R.). Spongy cadmium [16] increased analysis time. Nitrite was then determined by a modified Griess test [16].

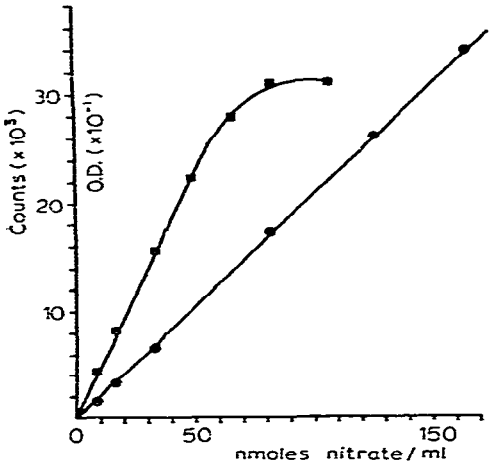
RESULTS

Comparison of nitrate analysis by HPLC and the cadmium reduction method

Unlike the HPLC method the cadmium reduction system was linear to only 60 nmol/ml of nitrate (Fig. 1). Furthermore, nitrate recovery from urine samples by the cadmium reduction method was less consistent than for HPLC analysis (Table I).

Identification of the nitrate peak

Three methods were used to verify the nitrate peaks. First, known amounts of nitrate were added to samples. Fig. 2 represents an example of this with rat urine. Figs. 2 and 3 have been drawn from computer displays; the blank areas immediately following injection represent void volume peaks not included in the figures. Fig. 2C shows a typical elution profile of nitrate (16.1 nmole) in distilled deionized water. Figs. 2A and B are elution profiles of the same urine sample except that 2A has been spiked with 16.1 nmole of nitrate. The sum of the nitrate peak areas in Fig. 2B and C equals the area (amount of nitrate) in the peak of 2A indicating the efficiency (98.6%) of nitrate recovery by this HPLC method. In similar experiments, with over 40 different urine and fecal samples, the mean difference between added and recovered nitrate was 2.83% (standard deviation: 2.61).



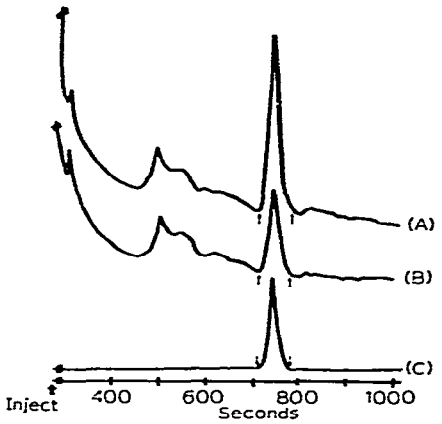


Fig. 1. Comparison of analysis of nitrate in distilled deionized water by the cadmium reduction method and HPLC. Circles represent HPLC (counts) and squares represent cadmium (O.D.) analysis of nitrate.

Fig. 2. Representative example of a rat urine (A) after and (B) before addition of a standard nitrate spike (16 nmol/ml). The same standard spike is shown in (C) in distilled deionized water. These three separate analyses have been stacked to allow better comparison of the results. The first part of each trace has been omitted. Arrows indicate computer marks used for nitrate peak area integration; counts in A = 7324, B = 3490, C = 3943. Flow-rate = 1.5 ml/min.

TABLE I

ACCURACY OF THE CADMIUM REDUCTION METHOD AND HPLC FOR THE ANALYSIS OF NITRATE IN RAT URINE

	Cadmium			HPLC		
	O.D. Observed	O.D. Expected	Recovery (%)	Counts Observed	Counts Expected	Recovery (%)
Sample (S)	0.023	N.A.*	N.A.	1299	N.A.	N.A.
S + 1.6 nmol/ml of nitrate	0.100	0.112	89.3	1569	1594	98.4
S + 8.0 nmol/ml of nitrate	0.395	0.417	94.7	3000	2922	102.6
S + 16.1 nmol/ml of nitrate	0.675	0.831	81.3	4396	4405	99.8

*N.A. = not applicable.

Secondly, nitrate peaks were identified by cadmium reduction of effluent fractions followed by azo dye determination of nitrite [16]. Assignments agreed with those based on spiking experiments (data not shown).

The third method was conversion of nitrate to ammonia with Devarda's alloy. The peak bounded by arrows in Fig. 3A was not present after alloy treatment (Fig. 3B) indicating it was nitrate.

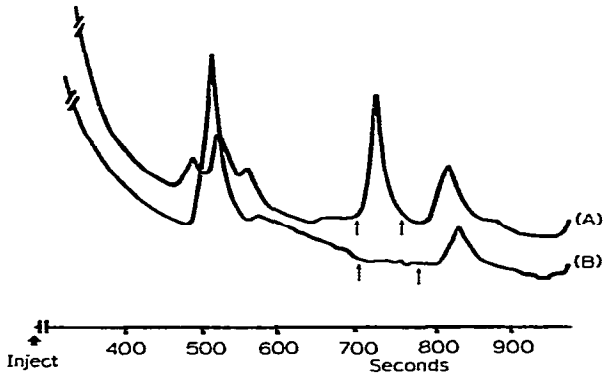


Fig. 3. Representative example of HPLC effluents of a rat urine containing nitrate (A) and the removal of nitrate (B) by Devarda's alloy. The first part (left) of each trace represents the void volume and has not been reproduced. Arrows (at 700—800 sec) indicate computer marks used for nitrate peak area integration. Flow-rate = 1.5 ml/min.

Precision of the HPLC method

Precision was estimated by analyses of 52 urine and fecal samples in triplicate. The mean coefficient of variation was 1.91% (standard deviation 1.33).

DISCUSSION

HPLC analysis is superior to the cadmium reduction method for assay of nitrate in biological samples. Previously reported HPLC techniques [14,15] proved to be inadequate for quantitating nitrate in urine, feces and animal tissues. The modification described in this report allowed such quantitation. Rat blood, kidney and liver extracts as well as rat and human urine and feces and several foodstuffs were successfully analyzed for nitrate. Furthermore, this HPLC technique allowed the demonstration of nitrate synthesis by mammalian tissues in vivo [17].

Several practical points can be made for this HPLC method. Nitrate analysis times were 15—25 min depending on buffer pH (and to a lesser extent on ionic strength) and on the presence of compounds eluting after nitrate. More acidic buffers gave shorter retention times. Chloride, but not sulfate, adversely affected resolution of the nitrate peak. There were only minor differences in the nitrate peaks obtained with Partisil SAX or Ultrasil AX columns. Both columns gave good service for over a year. Guard columns packed with HC Pellosil were superior to those packed with Partisil. Comparison of raw samples with those treated with Devarda's alloy greatly helped in correct assignment of nitrate peaks.

In conclusion, this report describes a procedure for nitrate analysis of complex biological samples which is safe, rapid and direct. It can detect 0.8 nmol/ml with a precision of better than ±5%.

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